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Patterns of DNA Replication of Human Chromosomes. II. Replication Map and Replication Model

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SUMMARY

Combining higher resolution chromosome analysis and bromodeoxyuridine (BrdU) incorporation, our study demonstrates that: (1) Human chromosomes synthesize DNA in a segmental but highly coordinated fashion. Each chromosome replicates according to its innate pattern of chromosome structure (banding). (2) R-positive bands are demonstrated as the initiation sites of DNA synthesis in all human chromosomes, including late-replicating chromosomes such as the LX and Y. (3) Replication is clearly biphasic in the sense that late-replicating elements, such as G-bands, the Yh, C-bands, and the entire LX, initiate replication after it has been completed in the autosomal R-bands (euchromatin) with minimal or no overlap. The chronological priority of R-band replication followed by G-bands is also retained in the facultative heterochromatin or late-replicating X chromosome (LX). Therefore, the inclusion of G-bands as a truly late-replicating chromatin type or G(Q)-heterochromatin is suggested. (4) Lateral asymmetry (LA) in the Y chromosome can be detected after less than half-cycle in 5-bromodeoxyuridine (BrdUrd), and the presence of at least two regions of LA in this chromosome is confirmed. (5) Finally, the replicational map of human chromosomes is presented, and a model of replication chronology is suggested. Based on this model, a system of nomenclature is proposed to place individual mitoses (or chromosomes) within S-phase, according to their pattern of replication banding. Potential applications of this methodology in clinical and theoretical cytogenetics are suggested.

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INTRODUCTION

During the last two decades, [^{3}H]thymidine incorporation, followed by autoradiography, has been frequently used in the study of mammalian chromosome replication. Using human cells, it has been established, for example, that certain chromosomes or chromosomal regions replicate their DNA terminally in a synchronous fashion (literature reviewed in [1–2]). Later, Ganner and Evans [3] and Calderon and Schnedl [4], combining Q-banding and [^{3}H]thymidine incorporation, suggested a relationship between chromosome banding and patterns of DNA replication. Because of the limited resolving capacity of silver labeling, these studies left open several questions concerning the replication timing of G and Q bands.

The disadvantages of autoradiography were recently overcome by methods that permit detection of chromosomal incorporation of the thymidine analog 5-bromodeoxyuridine (BrdUrd) [5–9]. Consequently, these techniques and in particular the BrdUrd-Hoechst 33258-Giemsa method and its variants [9] have produced a higher resolving power allowing analysis of patterns of chromosome replication at the metaphase band level (reviewed in [10]).

Recently, we demonstrated that cell synchronization combined with BrdUrd pulsing and fluorochrome-photolysis-Giemsa (FPG) differential staining further increases the resolution of the analysis because (1) it permits the scrutiny of shorter intervals of the S phase, (2) sharper and finer band definition can be obtained, and (3) elongated premetaphasic chromosomes can be studied [11–12].

The purpose of this study is to apply our methodology in determining the relative replicational behavior of different types of chromatins such as: facultative heterochromatin, constitutive heterochromatin, Y-heterochromatin, G-bands (intercalary "heterochromatin"), and R-bands.

A replicational map has been established for every human chromosome, and, consequently, the model of replication chronology is presented. Finally, we propose a nomenclature that combines the ISCN [13] system with the replication pattern in five basic stages of replication. This system allows positioning of every mitosis or chromosomal band according to its replication timing within the S-phase.

MATERIALS AND METHODS

Cell Synchronization and BrdUrd-Labeling

Human peripheral lymphocytes from chromosomally normal individuals were cultured, synchronized, and pulsed with BrdUrd as described previously [11]. In essence, the method consists of releasing a methotrexate block with BrdUrd, and collecting mitoses at different intervals following a brief (15-min) Colcemid treatment. With this protocol (B-pulse or terminal BrdUrd-labeling), those chromosomal regions that had replicated before the addition of BrdUrd would be darkly stained.

Particular attention was focused on determining the replication pattern during transition between the end of replication of R-bands and the beginning of replication of G-bands. According to our previous experiments [11–12], this period can be clearly detected in those samples terminally labeled with BrdUrd for 5–6 hrs prior to harvest. Also, during this interval, the highest proportion of R-banded mitoses is obtained. Therefore, further delineation of this transition was achieved by collecting samples every 15 min within the range of 4½–7 hrs of BrdUrd treatment.

The so-called T-pulse method is the reverse of the BrdUrd design. The cells are released from 17 hrs of methotrexate-block by change of media with 30 μg/ml of BrdUrd, 0.1 μg/ml 5-fluorodeoxyuridine, and 2 μg/ml of uridine, and incubated for 3–4 hrs. Thymidine-enriched medium is substituted for an additional 3–4 hrs.

Slide Preparation and Staining

Cultures were treated with Colcemid (0.07 μg/ml) for 15 min, followed by the conventional hypotonic treatment with KCl (0.075 M) for 8 min at 37°C and 3:1 methanol-acetic acid fixation. The cells were washed twice with fixative and stored overnight at 4°C. The following day, cells were washed three times in fresh fixative and the cell suspension dropped onto methanol precleaned slides moistened with deionized water at room temperature. Slides were allowed to drain and dry at room temperature in vertical position and then stored in plastic boxes.

One week later, the slides were differentially stained according to a slight modification of the FPG method of Perry and Wolff [9] and Goto et al. [14]. Air-dried slides were stained for 5–10 min with Hoechst 33258 dissolved in deionized water. Last year we reported using a concentration of 50 μg/ml of this fluorochrome. However, since then we have used two new batches of this stain and found that the concentration and time had to be modified for better results. Optimal results were obtained staining the slides for 5–10 min with solutions three times more concentrated (150 μg/ml). After the Hoechst 33258 staining, the slides were briefly rinsed in deionized water, air dried, and mounted under a coverslip with 0.35 ml of McIlvaine's buffer, pH 7.0, and illuminated for 30 min with a 100-W spotlight mercury vapor lamp at a distance of about 25 cm, maintaining thus a temperature of 47°C–50°C at the slide level. We found this range of temperature critical, since temperatures below 45°C or above 52°C resulted in poor differentiation between bands (poor contrast). However, this temperature range might have been critical only under conditions in our laboratory, but under different circumstances the optimal temperature range might vary.

Once the coverslip was removed with the aid of a blade, the slides were subsequently rinsed in water, dried, and mounted again under a coverslip with 0.35 ml of 2×SSC (0.3 M NaCl plus 0.03 M trisodium citrate, 1:1), pH 7.0, and incubated at 60°C–62°C for 15–20 min and finally stained in a horizontal position for 3.5–4 min with 2% Giemsa solution in phosphate buffer at pH 7.2. To avoid stain precipitation in the slides, the Giemsa solution was prepared fresh every day and mixed well immediately before use.

Photographs and Stages of Replication

Mitoses were selected according to the length of the chromosomes, that is, premetaphasic spreads such as early metaphases and prometaphases were preferentially chosen and photographed.

Photographs were made using a 63×-oil objective and Kodak high-contrast copy film no. 5069, printed on Agfa Rapidoprint no. 1 or 2. Recently, we have tried other films with satisfactory results such as Kodak Technical Pan film 2415 and Kodalith Ortho-film.

The differentially stained mitoses were classified into five stages of replication based on criteria described in RESULTS. A total of 30–50 different copies of each of the 24 chromosomes (1–22, X, and Y) from different mitoses and subjects were individually cut out of prints, labeled in the back, and analyzed.

RESULTS

Stages of Replication

Since terminal B-pulse protocols were used throughout most of the study, by exposing the cells to increasingly longer periods of BrdUrd, the frequency of

darkly stained bands tends to decrease. Consequently, five different stages were identified and described as follows:

Stage I. Corresponds to those cells that have incorporated BrdUrd during the entire length of the S-phase, that is, one complete cycle of replication in BrdUrd. Every chromosome of the complement stains light without longitudinal differentiation or bands. Lateral asymmetry is commonly observed in chromosomal regions containing large segments of C-heterochromatin such as in chromosomes 1, 16, and Y but rarely on 9 (see fig. 1). The remaining chromosomes exhibit a small, dark dot placed exactly at the centromere. This structure does not correspond in size to the regular C-band.

Stage II. All chromosomes except the late-replicating X (LX) show a replicational pattern resembling an incompletely developed R-banding. It means that

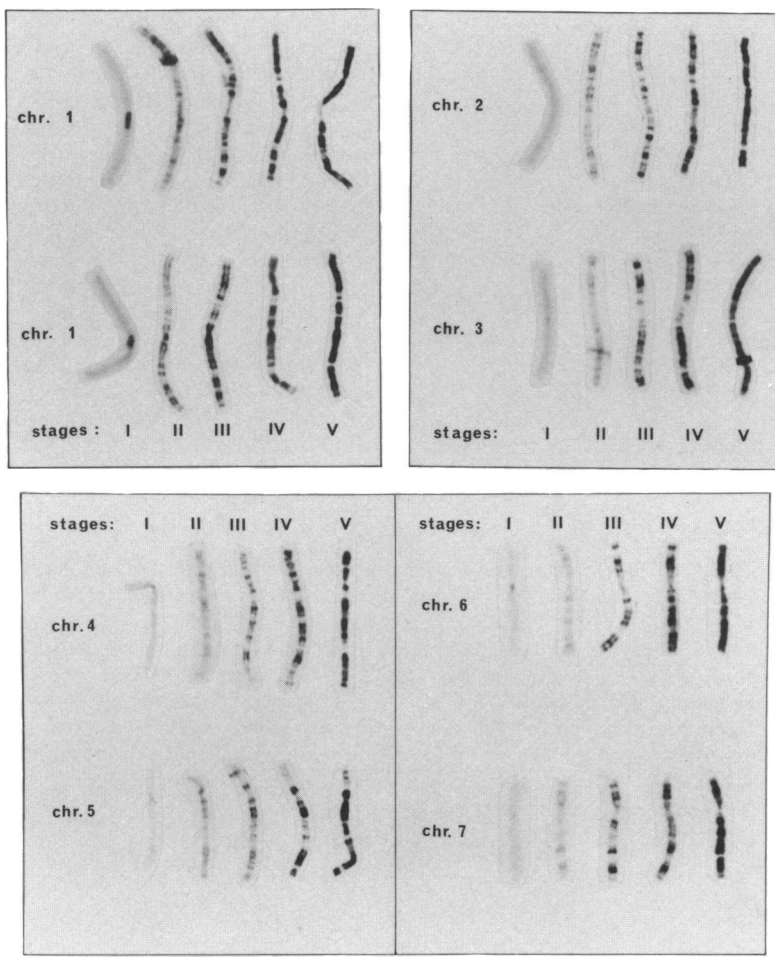


FIG. 1.—Replication map of human chromosomes represented in five stages of replication

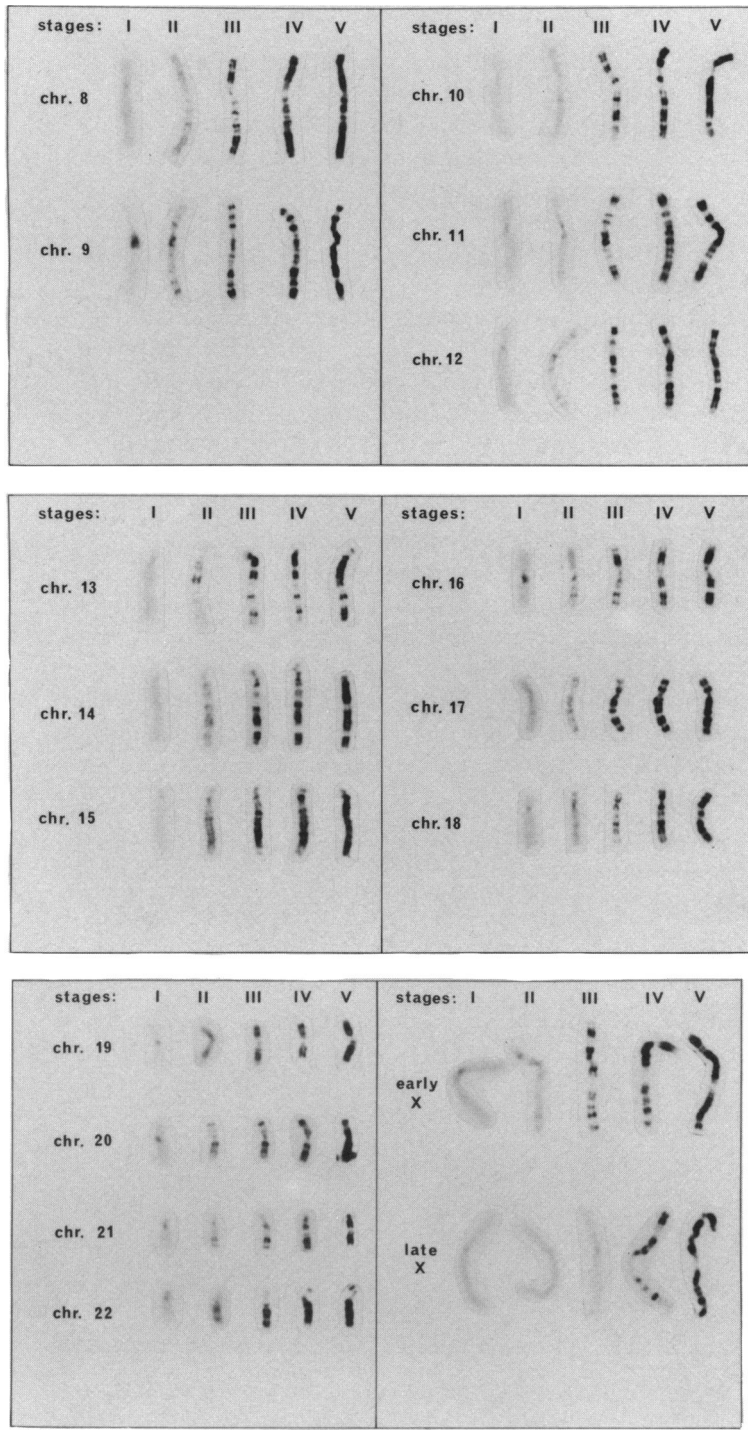


FIG. 1 (*continued*)

these regions already underwent partial replication in thymidine (T) prior to the time when BrdUrd was added to the culture. Therefore, Stage II is defined as an incomplete stage of replication of the R-bands, which become fully developed in the next stage. It seems likely that most, but not all, R-bands start replication simultaneously, during the first part of the S-phase.

Stage III. Autosomes present a replication pattern that clearly resembles "R-banding"; this also applies to the early-replicating X (EX). The late-replicating X (LX) remains totally pale or shows very few bands.

Stage IV. During this period, chromosomes retain some of the R-like pattern. However, a more detailed inspection reveals that: T-incorporation has extended from the already replicated R-bands to parts of the G-bands. The fine subbands within major bands disappear and fuse, forming large blocks of dark and wider bands. Several R-bands replicate in the LX, and at the end of this period, the lateral asymmetry on the Y chromosome tends to be replaced by a large late-replicating band. C-bands begin a rapid incorporation of T.

Stage V. Based on a combined analysis of terminal B-pulse and T-pulse experiments, we have revised our previous definition of Stage V, in the following manner: Short terminal BrdUrd pulses revealed that C-bands complete replicating during this period; this was also confirmed using T-pulse experiments that revealed that at least two fractions can be identified within the C-bands based on their replication chronology (fig. 2). Also during Stage V, some major autosomal G regions finish their replication. In the LX, the following bands are among the latest to finish replication in the entire complement: Xp21 and Xq21.

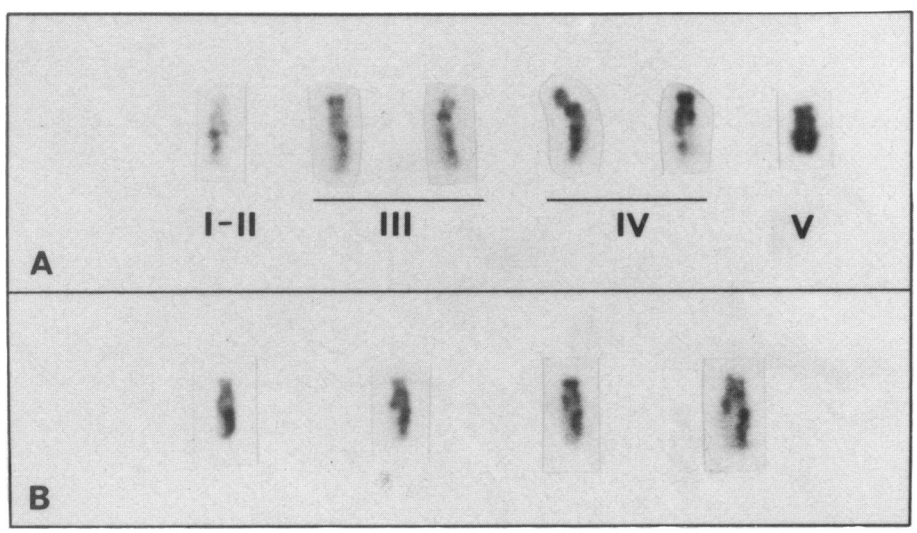


FIG. 2.—*A*, Stage III; *B*, Stage IV; *C*, Stage V; as revealed with the T-protocol (terminal thymidine pulse). Note the close resemblance between Stage III-banding and R-banding. *Right column* illustrates different replicational fractions of centromeric bands in chromosomes 1, 9, and 16 during the III-to-V transition.

TABLE 1

DISTRIBUTION (IN PERCENTAGE) OF STAGES OF REPLICATION AMONG MITOTIC SAMPLES COLLECTED AT 3–13 HRS AFTER THE RELEASE OF AN MTX BLOCK WITH BRDURD

HRS IN BRDURD	STAGES OF REPLICATION				
	I	II	III	IV	V
3	...	...	13.3 ± 3.1	46.8 ± 8.3	39.8 ± 7.3
4	...	...	33.2 ± 4.9	55.1 ± 6.3	12.2 ± 5.0
5	...	...	63.2 ± 8.5	37.4 ± 5.7	...
6	...	8.3 ± 3.0	75.9 ± 11.8	16.3 ± 5.3	...
7	5.6 ± 3.2	14.6 ± 3.0	74.2 ± 8.3	5.7 ± 3.9	...
8	20.4 ± 4.0	16.7 ± 3.6	63.2 ± 7.6	...	...
9	41.3 ± 6.0	16.6 ± 4.4	42.0 ± 7.7	...	...
10	54.8 ± 7.2	15.6 ± 4.0	28.9 ± 5.2	...	...
11	66.8 ± 7.1	13.4 ± 4.2	19.9 ± 4.0	...	...
12	74.8 ± 6.8	8.2 ± 3.5	17.3 ± 3.9	...	...
13	79.4 ± 7.7	4.0 ± 2.7	16.4 ± 3.3	...	...

NOTE: Each no. represents a 10-sample average ± SD.

Table 1 shows the average distribution of previously described stages of BrdUrd incorporation from samples of 10 chromosomally normal subjects.

Replication Chronology

The Autosomes. A detailed analysis of the replication map represented in figure 1 reveals the following: (1) Replication initiates simultaneously in many bands along each chromosome, and they correspond in position to R-bands (G-negative bands). About 85%–95% of the R-bands show evidence of T-incorporation during Stage II of metaphase chromosomes. However, if longer premetaphase chromosomes are used (fig. 1), the number of bands observed in Stage II is obviously higher. The most conspicuous initiation bands generally correspond to the major or larger R-bands, but only in the next stage do they become equivalent in size to conventional RHG-bands. (2) The maximum number of replication bands is obtained during Stage III. Again, they generally correspond in position to normal "structural" R-bands (ISCN, 1978). However, as we will discuss later (fig. 4), there are some exceptions. One of them is that more bands are consistently obtained with replication banding (B-protocol) than with RHG-banding (R-bands by heating using Giemsa) if chromosomes of a similar length or contraction are compared. The ISCN [13] was used for these comparisons. In summary, during Stage III, few additional bands appear. All R-bands finish their replication, and a sharp R-pattern is fully developed. (3) With the exception of several pericentromeric areas, during Stage III no detectable T-incorporation is observed in between R-bands, that is, in between the light spaces corresponding to G(Q)-positive bands. This indicates that a clear chronological demarcation exists between the replication of R- and G-bands as has also been confirmed using the T-pulse protocol. (4) G-bands initiate their replication during Stage IV and become fully replicated in Stage V. This is evidenced by a "filling" effect that commences in the fine G-bands. The visual effect under the B-protocol is that smaller R-bands seem to fuse.

This is because of T-incorporation in the G-bands. On the other hand, in some major G-bands, replication seems to progress from the already replicated R-bands toward the center of the unstained regions (G-bands). Two clear examples are seen throughout Stages III–IV–V in bands 1q31 and 12q21 (fig. 1). A possible explanation of this phenomenon is given in the DISCUSSION. (5) The latest autosomal bands to complete DNA-replication are the pericentromeric areas and some major G-bands such as 1q31, 5p14, 7q31, 12q21, 13q21, 14q31, and 21q21.

The Sex-Chromosomes (EX, LX, and Y). In normal XX cells, the *early-replicating X* chromosome (EX) behaves like an autosome. Therefore, the same replication characteristics described in the previous section (IIA) apply to the EX (fig. 1).

The *late replicating X* (LX) starts replicating at the beginning of Stage IV and occasionally at the end of Stage III. In analogy to autosomes and EX, G-bands replicate after R-bands with virtually no overlapping and R→G sequence demonstrated in the autosomes is conserved. G-bands p21 and q21 are the latest to complete replication (fig. 1). This observation was also confirmed with the T-pulse methodology (fig. 2).

In normal XY cells, *the Y chromosome* shows the following replicational behavior (fig. 3): (1) Lateral asymmetry in band Yq12 is detectable throughout Stages I–IV, which indicates that this large heterochromatic segment replicates during Stage V, thus being one of the latest-replicating regions of the genome. (2) During Stage II, the Y chromosome remains practically unchanged, but in the next stage (III), R-bands Yp11 and Yq11, that is, the so-called euchromatic portion of the Y, show evidence of T-incorporation. At this point it is important to note that according to the ISCN [13] the nonheterochromatic part of the Y chromosome should be composed entirely of R-positive material. However, when R-banding techniques are used, these regions contain a rather weak R-positive material intercalated with some fine G-positive bands. Similarly, with replication banding, these R-bands are visible at the end or after Stage III. (3) The nonheterochromatic portion of the Y chromosome becomes fully replicated during Stage IV. The small G-positive bands intercalated within Yp11 and Yq11 complete their DNA synthesis by the end of this stage.

The replication chronology of the Y chromosome can be graphically summarized as depicted in figure 3*A*. A common feature of late-replicating elements such as the Y and the LX is that a rapid succession of events takes place during the III–IV transition. Therefore, it is expected to see some intermediate patterns as depicted in figure 3*A*.

During Stage III, different forms of lateral asymmetry are occasionally observed in the nonheterochromatic band Yq11 (fig. 3*B*). The main characteristic here is that the distal half of band Yq11 (q11.2-q11.3) is preferentially involved. Compounded lateral asymmetry has also been observed apparently involving the entire band Yq11. In the next section, we will discuss a possible explanation for this phenomenon (fig. 3).

Autosomal C(+) Regions. There is little doubt that autosomal constitutive heterochromatin contains late-replicating elements. But questions such as "where during S-phase does the C-heterochromatin commence and complete replication"

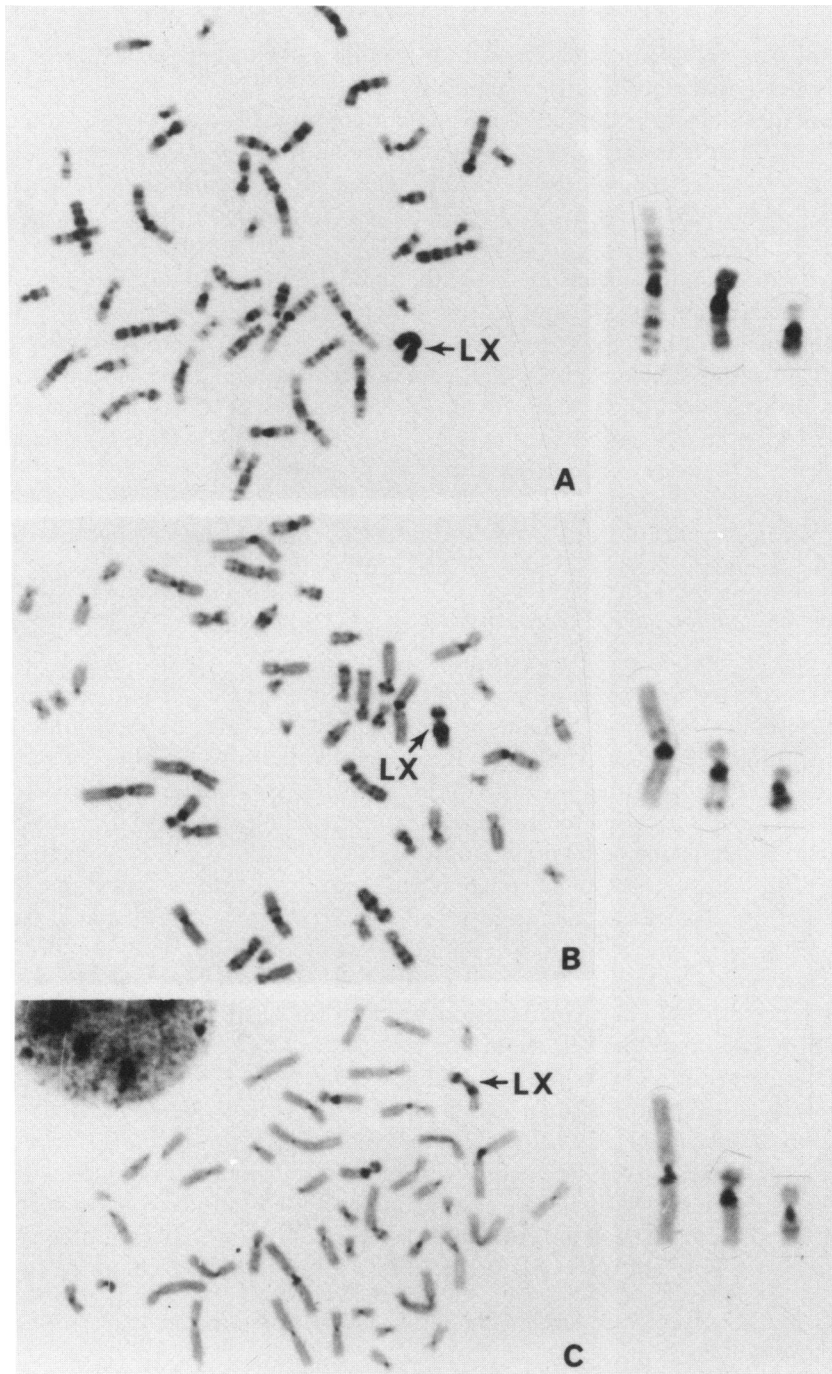


FIG. 3.—*A*, Representative samples of replication Stages I–V in normal human Y chromosomes. Note the persistence of lateral asymmetry throughout Stages I–IV. *B*, Examples of compounded lateral asymmetry in the "euchromatic" Yq11 segment. *From left to right*, 0 to 3 exchanges.

or "how many replicational classes of C(+) bands can be distinguished" remain to be answered and require further analysis.

These questions were approached using both terminal T- and B-pulse protocols. Terminal T-pulses were particularly useful in the study of the very terminal sequences of replication, especially of the autosomal pericentromeric regions (C-bands) because they appear as sharp dark bands against a light chromosomal background (fig. 2).

Our findings indicate that DNA synthesis in C(+) regions starts during Stage IV, proceeds at a high rate, and ceases at the end of Stage V. During early Stage IV, centromeric bands closely resemble C(+) bands in shape and position (fig. 2*A*). However, as this stage progresses, at least two replicational fractions of C-bands can be distinguished. In fact, during the IV–V transition, a consistent reduction in size in *all* pericentromeric bands can be noticed (fig. 2*B–C*). This is particularly evident in chromosomes 1 and 16.

From these results, it can be concluded that C(+) regions are composed of at least two late-replicating fractions (C_{IV} and C_V), one of them being among the latest to terminate DNA synthesis in the entire genome.

Replication Banding and Nomenclature

The above defined five basic stages of replication clearly place any particular cell in its correct position in S-phase independently from the time of observation. Distinct replicational events have been used to demarcate the replicating time zones within the S-phase. Therefore, it is suggested that this simplified classification could be incorporated into the ISCN [13] whenever the terminal BrdUrd or T-pulse protocols are used to obtain replication banding and/or to describe chromosomal rearrangements or breakpoints.

For instance, in cases of terminal B-pulse protocols, any particular banded karyotype or cell could be identified as follows: *BHG (Stage III-banding* or *(MTX) BHG (Stage IV)-banding*. This designation would indicate that the BrdUrd-Hoechst-Giemsa technique was used to produce Stage III or Stage IV banding without or with MTX synchronization, respectively. In the case of terminal T-pulse protocols, only the "B" would be changed to "T," that is, to THG (Stage III) banding or (MTX) (Stage IV)-banding.

Similarly, breakpoints could be easily identified as recently demonstrated in two cases of retinoblastoma [15]: 13q14:q21 (BHG) (Stage III). In summary, the recommendations of the ISCN [13] are followed but new information is added with the statement on the replication stage and the method used to induce cell synchrony. Suggested nomenclature should simplify the description of "replication banding" obtained with different protocols (BrdUrd or T) and staining techniques.

Comparison between Replication Bands (Stage III) and Structural Bands (RHG)

Previously, we indicated that under terminal B-pulse protocol, Stage III mitoses show a banding pattern that resembles R-banding. To recap, Stage III is the most informative replicational stage because it shows the highest number of bands,

allows detection of facultative heterochromatin (LX), the unequivocal differentiation of Y chromosomes, regions of lateral asymmetry, and, above all, sharp band definition. No single banding technique that we are aware of presents all these combined advantages. Stage III is also the most abundant stage among mitoses collected 5½–6 hrs after BrdUrd release of an MTX block.

Therefore, we considered it useful to study in detail the Stage III banding pattern. For this purpose, average early metaphasic diagrams were constructed for each chromosome (fig. 4). Such idiograms present both the comparative analysis with standardized R-bands [13] and the observation of the beginning of prometaphase subbanding.

Stage III mitoses resemble R-banded karyotypes, and, as in regular GTC and RHG banding, not all bands stain with equal intensity. This staining gradation is represented by the use of two intermediate tones plus black and white (fig. 4). Additional tones seem to exist, but at this point their graphical representation is technically impractical.

We interpret this gradient in band intensity as a true reflection of the underlying fine subbanding contained within major metaphase bands and interbands. This is further supported by studies using prometaphase replication banding [16].

Stage III replication banding also shows improved resolution when compared with RHG-banded chromosomes of similar length. Apparently, chromatid details are better preserved under FPG staining than with RHG where 86°C–87°C hot saline is used.

The numbering system used in these early metaphase idiograms follows the Paris Conference (1971) recommendations. It is also important to emphasize that these idiograms represent an intermediate stage of chromosome contraction. Therefore, special attention was focused on determining the correspondence of each band and subband. A higher resolution analysis of prometaphase replication banding is discussed in [16]. Our findings lead us to propose the following replicational model (fig. 5). This model agrees not only with our experimental data but is also compatible with a considerable number of previous observations as will be discussed later. Different replicational events of the S-phase have been dissected including the initiation of DNA synthesis during early S, the R→G transition, the rapid synthesis of constitutive heterochromatin, G-bands, late-replicating X chromosome, Y chromosome, and patterns of lateral asymmetry. For the sake of clarity, each will be discussed individually.

DISCUSSION

Initiation and Early Events

Our data indicate that R-bands are the segments in which DNA replication is initiated at the onset of the S-phase as demonstrated on metaphasic and premetaphasic chromosomes (fig. 1). This finding agrees with recent data on diploid human fibroblasts (WI38) synchronized with FdUrd [17].

R-bands constitute the basic units of initiation of DNA synthesis. We found this initiation to occur in synchrony throughout the R-bands, a finding that is dispar-

ate with previous reports. The following observations strongly suggest that the so-called cascade or ordered sequence of initiation events reported by other investigators may be attributed to the limited resolution of the employed techniques. For instance, it is known that metaphase bands have different sizes and, presumably, contain different numbers of replicons. If we consider that a critical number of BrdUrd or T substitutions are required before they can be detected cytological-

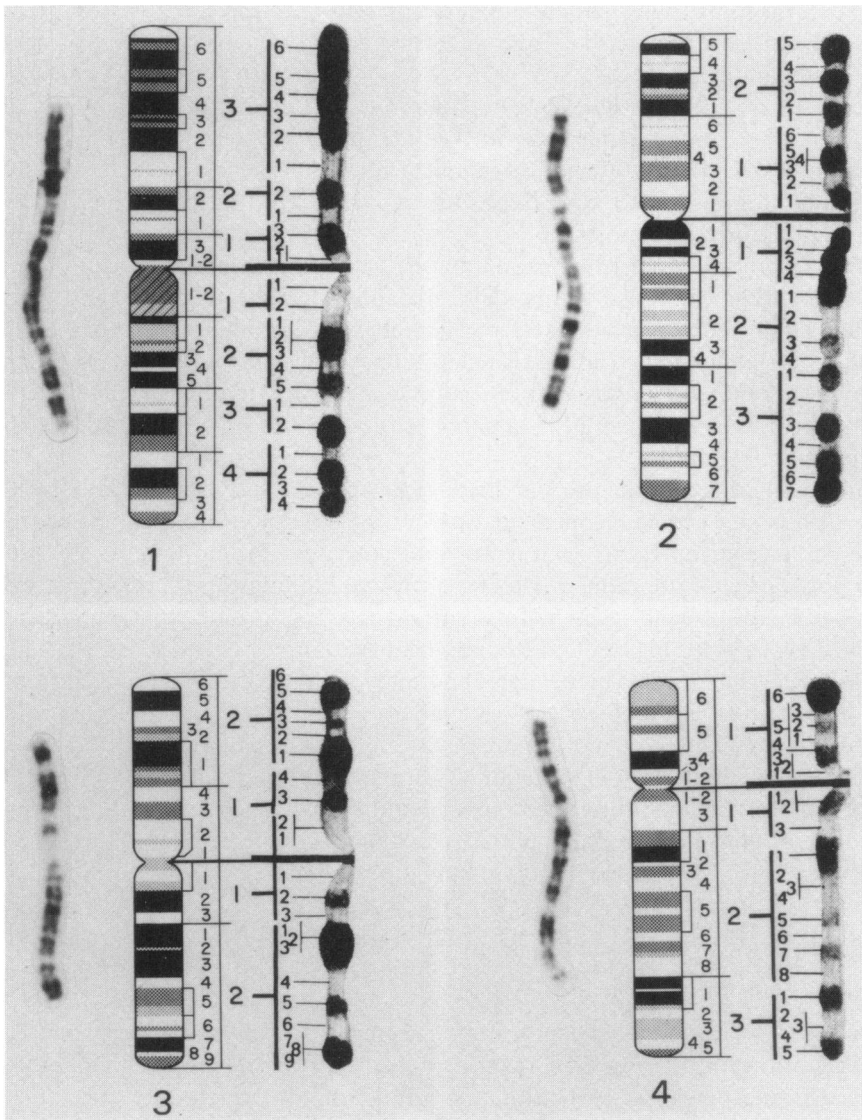
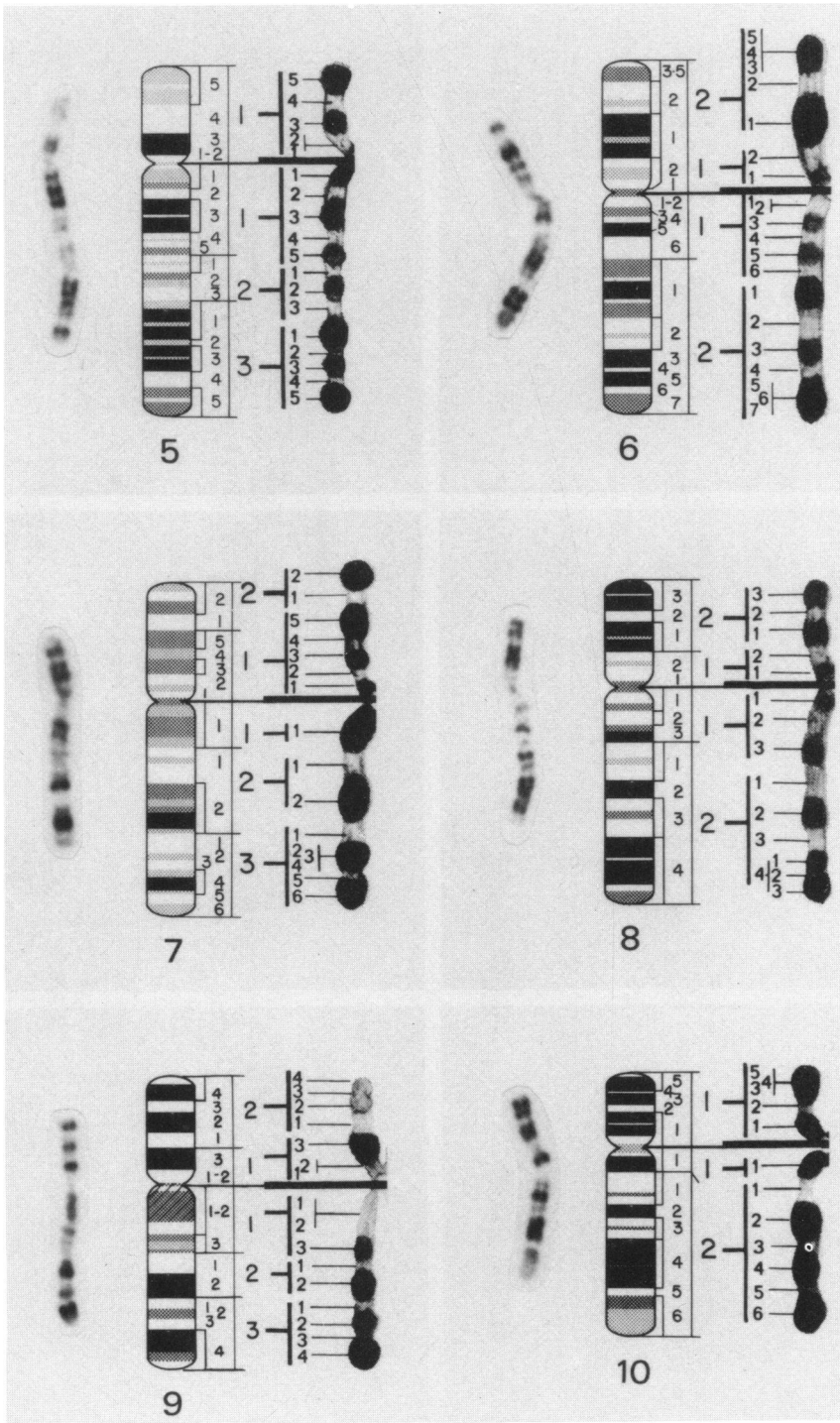


FIG. 4.—Replicational banding (*left and center*) compared with standardized ISCN (1978) R-banding (*right*) in human chromosomes 1–22, plus X. Numbering system proposed for replication bands (*center*) follows the Paris Conference (1971) recommendations.



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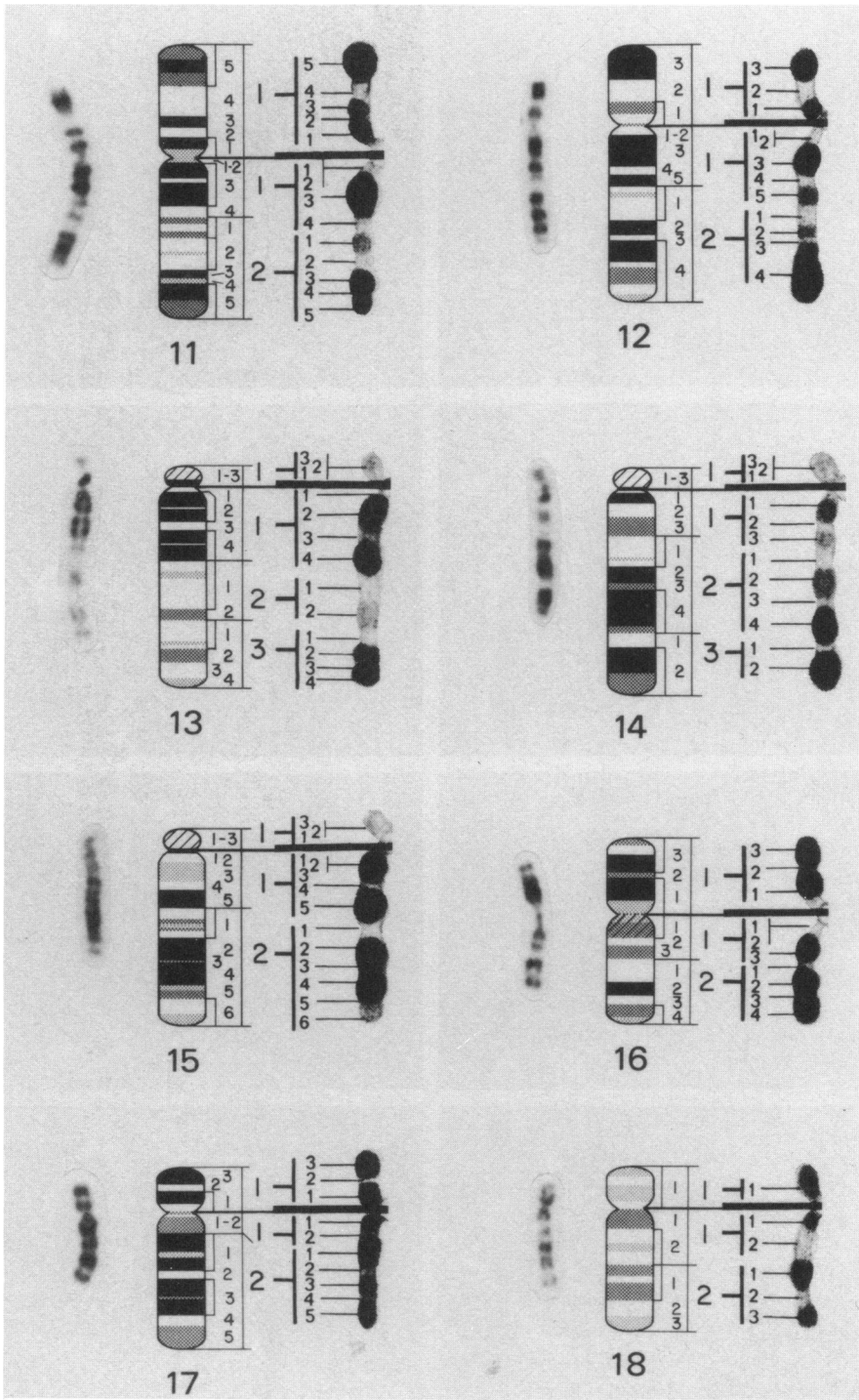

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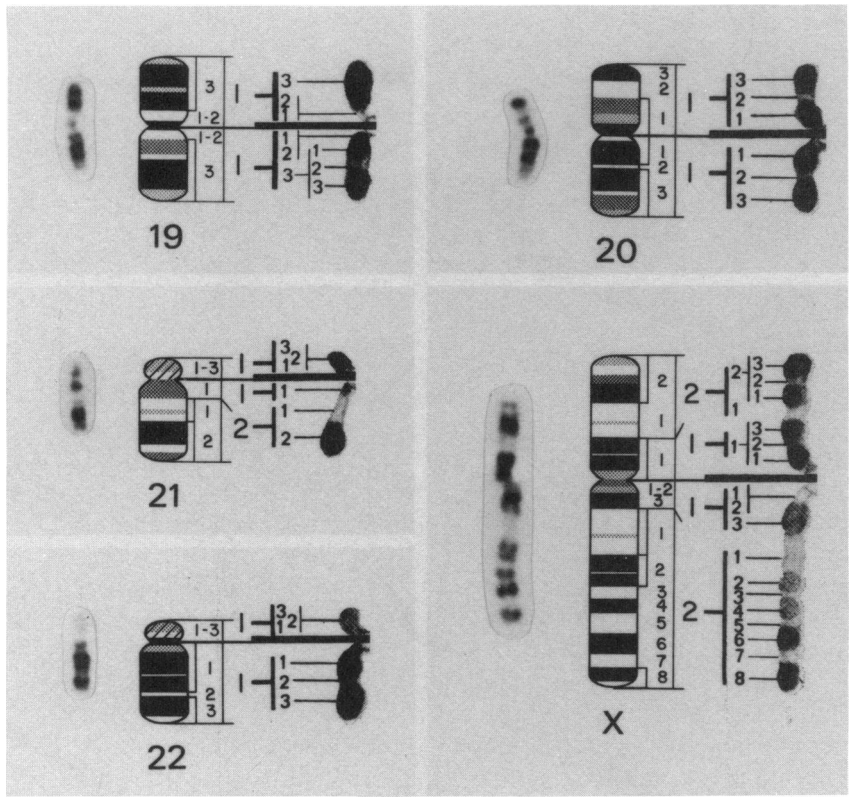

FIG. 4 (*continued*)

ly, it becomes clear that some bands may appear to replicate sooner than others. In fact, Dutrillaux et al. [18] reported that among R-bands, the most intensively stained ones are often those that appear to replicate earlier.

On the other hand, most studies reporting a "cascade" of initiation events involved nonsynchronized cell populations where BrdUrd pulses are often administered without a previous reduction or depletion of the endogenous de novo production of T. Under such conditions, it is expected that both BrdUrd and T are

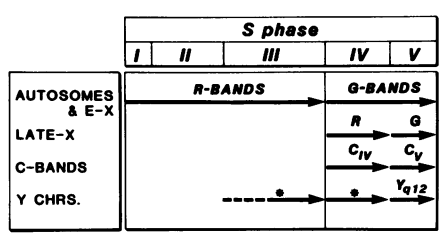

FIG. 5.—Replication model of human chromosomes: the replicational chronology of different chromatin types is indicated. (*See the text for a more detailed description on the Y chromosome.) *E-X*: early-replicating X chromosome.

simultaneously incorporated and/or incorporated at different rates. The preferential incorporation of one or the other may ultimately depend on the replication kinetics of each particular band that, in turn, may be merely a reflection of the number of replicons per band or the size of the band itself.

Metaphase bands are generally composed of subbands or a mixture of G and R subbands. Consequently, those chromosomal segments "richer" in R-bands (i.e., with fewer intercalated G subbands) might simulate earlier-replicating regions more likely than those segments relatively "poorer" in R-bands (i.e., major G-bands). A good example of the first type is shown in figure 1 (Stage II) in chromosomal regions 1p2, 11p2, 15q2, 17q2, and 22q1. Part of this problem that is inherent to the limited resolution of metaphasic chromosomes has been solved by analysis of prometaphase mitoses [16].

S-phase Subdivisions and the R→G Transition

Previous studies suggested that early-replicating regions complete DNA synthesis before the late ones start [18–19]. Further, it has been proposed that G(Q)-negative bands correspond to early-replicating segments and G(Q)-positive bands to late-replicating segments (reviewed in [10]).

However, previous attempts to subdivide the S-phase or to study the R→G replicational transition met with methodological difficulties, primarily as a result of the use of nonsynchronized cells. Variation in cell cycle and phase times (as would be expected in asynchronous populations) [20] confounded these attempts, as did the intermixture of T with BrdUrd-incorporation.

In our study, these problems have been overcome by inducing partial cell synchronization and reducing the endogenous production of T before the addition of BrdUrd. As a result, clear demarcation between T- and/or BrdUrd-incorporation was obtained [11–12]. With this improved resolution, we could not observe overlap between the replication chronology of R(+) and G(+) bands. Supporting data have been reported by Schmidt [21].

One clear implication of our findings and the model proposed is that R-bands constitute the only truly early-replicating segments in the autosomes and EX. G-bands, in contrast, display replicational features that clearly classify them as a distinct type of heterochromatin, that is, "G(Q)-heterochromatin." Other properties exhibited by G-bands including relative AT-richness and presence of moderately repetitive DNA reinforce this concept [10]. Therefore, it is no longer appropriate to refer to "early- or late-replicating autosomes" because they all contain a mixture of both early- and late-replicating segments.

Replication of Y Chromosome

Previous investigations employing autoradiographic techniques concluded that the Y chromosome is late-replicating. However, because of the nature of autoradiography, conflicting data were generated by other researchers and the question remained unresolved (early literature reviewed in [1–2]).

More recently, Craig and Shaw [22] and Knight and Luzzatti [23] suggested a replicational difference between the short arm of the Y and the rest of this chromo-

some. The combination of autoradiography and Q-banding [3–4] further supported this concept. However, results were again complicated by the coarse localization of autoradiographic "grains."

Most recently, the late-replicating nature of band Yq12 (Y-constitutive heterochromatin) has been clearly established using BrdUrd-incorporation and differential staining techniques [24], but the replicational behavior of Y-"euchromatin" remained poorly investigated.

Our results demonstrate that the G-negative portion of the Y chromosome (Yp11 and Yq11) is late-replicating and contains different elements or chromatin types whose replicational features can be visualized during the last third of the S-phase. For instance, it is clear that both Yp11 and Yq11 contain at least two subbands each, one which replicates at the end of Stage III (Yp11.2 and Yq11.1) and the others during Stage IV (Yp11.1 and Yq11.2). These findings imply that the so-called euchromatic portion of the Y chromosome is composed of both late- and early-replicating elements.

Several factors can account for this phenomenon: (1) The short arm of the Y chromosome seems to be composed of a mixture of fine R- and G-bands. In fact, in G-banded metaphasic preparations, Yp shows neither a light nor dark appearance but an intermediate one. Prometaphase studies have supported this suggestion [16, 25–27]. Therefore, we believe that the intermediate staining of the Yp can be explained by its structural composition. It contains chromatin that becomes visualized by the FPG technique only when fully replicated, that is, at the end of Stage III. Later, at the beginning of Stage IV, it appears more conspicuous as a result of the rapid incorporation of T residues that takes place during replication of the G-subcomponents.

(2) The centromere is obviously late replicating because it contains C-heterochromatin [28].

(3) Finally, the so-called "euchromatic" portion of the long arm (Yq11) presents more complex features. It is also composed of intercalated G and R subbands and contains repetitive DNA. In fact, Gosden et al. [29] documented that region Yq11 contains moderate amounts of satellite DNAs II, III, and IV. Similarly, Schmidtke and Schmid [30] reported that of the two male-specific reiterated sequences revealed by Hae III restriction endonuclease analysis (3.4 kb and 2.1 kb) the 3.4-kb sequence is possibly dispersed in the euchromatic region of the long arm. These findings would explain not only its late-replicating behavior but also the presence of lateral asymmetry in the C(−) (Yq11) segment of the long arm (fig. 3*B*). We find that this lateral asymmetry usually involves the distal half of band Yq11 and occasionally the entire long arm (fig. 3*B*). Comings [31] recently commented that not all repetitious DNA is C(+) and apparently this is one example. On the other hand, our observation of lateral asymmetry (LA) in a C(−) region of the genome (i.e., Yq11) complements the recently reported LA at the junction between Yq11 and Yq12 [32]. Emmanuel [33] also demonstrated LA in a C(−) region of the human genome (6q12→6q14).

The ability to differentiate segments of Y-"euchromatin" by replication banding presents potential applications in clinical cytogenetics, particularly in cases in

which only a fragment of a sex chromosome is present (Camargo and Cervenka, in preparation).

Finally, because of the close resemblance in replicational patterns between the short arm of the Y chromosome and the terminal portion of the EXp (bands Xp22.2), we will comment on the possible homology between these two regions. Moses et al. [34], for instance, analyzing spreads of human spermatocytes, found that the X-Y bivalent shows meiotic pairing and synaptonemal complex between the short arm of the Y chromosome (Yp) and approximately 1/5 of the distal segment of the Xp arm. In mitotic chromosomes also, the Yp arm is about the size of the Xp22.2→Xpter segment. Therefore, these two regions show not only close homology during meiosis but they are also equivalent in size and replicate in a similar fashion. The latter implies that they must contain similar types of chromatin. However, up to now there is no solid evidence of *genetic* homology between these two portions of the genome. Detailed gene mapping studies should be able to solve this paradox.

Autosomal C(+) Regions

Centromeric heterochromatin as demonstrated by the C-band technique is a very heterogeneous chromatin type and may be subdivided into a number of categories when other characteristics are considered [35]. Heteromorphisms in size and staining affinity are revealed using different techniques. In fact, various base-specific DNA binding agents have been used to differentiate types of C-heterochromatin in humans [36]. They include fluorochromes such as *quinacrine* [37], *Hoechst-33258* [38], *DBP* [39], *DAPI* [40], *olivomycin* [42], *DIPI* [41], *chromomycin* A_3 [40], and *mithramycin* [43].

Giemsa techniques also reveal differences among chromosomal regions rich in constitutive heterochromatin: among them, banding techniques such as CBG [44], GTG [45], and the G-11 method [46–47]. For instance, chromosomal regions corresponding to C(+) bands may stain light under GTG-banding (e.g., 9h+) or dark (e.g., 1h+) or intermediate (e.g., Yh+).

Although the mechanisms of these staining techniques are not entirely understood, they appear to relate to the underlying chromosome structure and DNA composition as strongly suggested from immunofluorescent and hybridization studies [29, 48]. For instance, the G-11 technique is thought to stain primarily satellite III DNA.

Therefore, it is not surprising to discover that C(+) regions contain distinct replicational fractions since *chromosome structure* and *replication chronology* are intimately related, as previously shown for R- and G-bands (*S-phase Subdivisions and the R→G Transition*). With replication banding, we identified three different fragments within C(+) regions and propose the following nomenclature: C_I-bands (Stage I, B-pulse), C_{IV}-bands (Stage IV, T-pulse), and C_V-bands (Stage V, T-pulse).

It is believed that C_I-bands reflect an uneven distribution of T between the two DNA polynucleotide chains [49–50]. This strand bias in T content can be manifested as regions of lateral asymmetry (LA) (e.g., in chromosomes 1, 15, 16, 17,

and Y) [51–52], also as asymmetrical bands such as in chromosome 9, or centromeric "dots" as in most of C-group chromosomes (fig. 2, first column).

In the latter case, the strand bias in T may not be present in these regions in a sufficiently long stretch to be cytologically detectable as LA by the present staining techniques [53]; the DNA composition or the internal distribution of satellite DNAs in these regions is another possibility [31]. Thus, we concluded that C_I-bands are present in *all* human chromosomes, they reveal regions of relatively high T-content, and represent a subset within centromeric heterochromatin. Their equivalence with repetitive or satellite DNAs is likely although speculative at the present time.

C_{IV}-bands (Stage IV, T-pulse) closely resemble standard C-bands as defined by the CBG-technique, and their late-replicating behavior is clearly demonstrated in this report (fig. 2). Previous experiments with unsynchronized human cells have resulted in photographs of similar patterns of replication [54–55]. Recently, Kondra and Ray [19] reported obtaining a banding pattern that simulates C-bands after a 5-hr terminal T treatment in unsynchronized fibroblasts. Our results confirm these observations and clearly define the onset and termination of C_{IV}-bands replication within the last third of S-phase.

Finally, C_V-bands (Stage V, T-pulse) represent a fraction of C_{IV}-bands. In major heterochromatic areas such as in chromosomes 1, 9, and 16, their size is about 1/3–1/2 of C_{IV}-bands, and in most chromosomes they appear as small, centromeric "dots." Centromeric heterochromatin in chromosome 1, for example, comprises bands q11 and q12, but under C_V-banding, it is restricted to the small 1q11 band. This type of fine replicational subdivision within constitutive heterochromatin, to our knowledge, has not been reported before in human cells. Its discovery is largely attributed to the increased resolution attained with the present technique.

Based on studies in animal species with large heterochromatic segments, there is sufficient reason to believe that C(+) regions indeed contain different replicational fractions. For instance, Popescu and Di Paolo [56], working with somatic chromosomes of Syrian hamster (*Mesocricetus auratus*), recently demonstrated that centromeric and noncentromeric heterochromatin are two distinct categories of constitutive heterochromatin. Similarly, substructures of C-band regions have been found in chromosomes of the kangaroo rat (*Dipodomys ordii*) after one complete S-phase in the presence of BrdUrd [57].

Summarizing, replication banding proves to be capable of identifying different chromatin fractions within constitutive heterochromatin. Further research is necessary to determine if these fractions may reflect a particular type of base sequence organization and/or their correspondence with C-heteromorphisms as identified with staining techniques.

Biphasic Replication: Additional Comments

Several investigators have noted that heterochromatin replicates its DNA more rapidly than euchromatin [58–61]. Comings [59], for example, demonstrated that the speed of replication is greater for heterochromatin than for euchromatin, and

thus the former one completes DNA synthesis in a shorter period of time. Our results indicate that this is the case for both facultative (LX) and constitutive (C-bands) heterochromatins and also for autosomal G-bands (G/Q-heterochromatin). They all replicate within the last third of the S-phase. The fast sequence of events that occurs during Stages IV–V provides clear evidence of their rapid synthesis.

Previous biochemical and autoradiographic (DNA fiber) studies also correlate with these observations. Painter and Schaeffer [62], for instance, have shown that HeLa cells in early S-phase have slower rates of chain growth than those in mid-S-phase. In CHO cells also, the rate of synthesis in early-S is about 650 bp/min [63]. A bimodal distribution of DNA synthetic activity has been reported in diploid mammalian cells [64].

These observations, in conjunction with the evidence presented here, provide strong support to the biphasic mode of replication in mammalian cells. Additional evidence includes changes in buoyant density (GC-to-AT shift) between early- and late-replicating DNA and the well-established correlation with R- and G-bands that contain GC-rich and AT-rich DNA, respectively (reviewed in [10]).

In spite of the clear chronological demarcation between early (R)- and late (G)-replicating chromatin, some exceptions have been reported [61, 65–69]. The following methodological factors could have contributed to these results: (1) Cells are analyzed in a noninformative stage of replication. For instance, if the BrdU-pulse is too short, G-bands may result in only partial labeling and hence appear as if they replicate with R-bands. (2) Deficient separation between T- and BrdU-pulses. This, as indicated earlier, occurs when BrdU is applied terminally without depletion of endogenous T production in asynchronous populations. (3) In quantitative autoradiography, chromosomes are often divided into equal segments and the number of grains scored independently from the underlying banding pattern.

It has been proposed that specific DNA base sequences (e.g., palindromes) and/or conformational changes in chromatin structure may govern initiation and temporal order of DNA synthesis. However, the chronological priority of R-band replication followed by G-bands within the late-replicating X chromosome imposes additional restrictions to these models. Therefore, they will remain speculative until the physical nature of replication origins in early- and late-replicating mammalian DNA (chromatin) is elucidated.

CONCLUSIONS

This investigation confirms not only that chromosomal replication is tightly linked to chromosomal banding but also shows that this methodology is a potentially valuable technique because it: (1) produces sharp band definition and increased resolution, that is, allows detection of BrdUrd-incorporation in early metaphase and prometaphase subbands; (2) differentiates not only the inactivated X chromosome from the rest of chromosomes but also segments of the Y chromosome; (3) can be used to cytologically study "spreading effect" particularly in cases of unbalanced X-autosome translocations and X-Y translocations; (4) reveals chromatin subfractions, particularly through patterns of lateral asymmetry and/

or terminal BrdU/T-pulses; and (5) allows subdivision of the S-phase according to precise patterns of chromosome replication or cytologic mapping of replicational events.

Our results also suggest the use of Stages III and IV in clinical cytogenetics not only because they present clear band definition and most of the features previously outlined but also because they are the most numerous and informative stages collectable 5½–6 hrs after the release of the MTX block. Finally, a replication model and nomenclature is proposed that could be applicable to other mammalian species.

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