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Computer Image Analysis of Variance between Human Chromosome Replication Sequences and G-Bands

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SUMMARY

A computer image analysis system was applied to the quantitative study of chromosomal early- and late-replication patterns from the leukocytes of several normal human donors, and these patterns were compared with the chromosomal G-banding patterns. The first and last few hours of replication were discriminated by selective bromodeoxyuridine vs. thymidine incorporation in DNA and a Hoechst-blacklight-Giemsa stain technique. Image analysis with the Tufts Piquant system involved automatic determination of chromosome boundaries, centromeres and telomeres, linear chromatid axes, chromatid density measurements along each axis, and comparative length normalized density profiles for each chromatid and the chromosome. Consistent complementary early- and late-replication patterns were determined for autosomes 1–6 and the X chromosomes. Limited intracellular or interindividual variability occurred in the intensity of a few active replication peaks but not in their location. However, there were very distinct regions of noncorrespondence between the late-replication patterns and the G-band patterns, in contrast with previous observations, although many similarities were also evident. These differences are interpreted with reference to a general model of replication sequence control of cell differentiation.

INTRODUCTION

Shortly after Q-banding had been discovered, Ganner and Evans [1] demonstrated with autoradiographic techniques that the banding patterns largely correlated

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with the late-replicating regions in human lymphocyte chromosomes. This early study thus initiated the idea that Q- and G-positive band regions reflected late-replicating, adenine-thymidine (A-T)-rich DNA [2]. Initial studies applying the new bromodeoxyuridine (BrdU) incorporation sister chromatid exchange staining techniques to the study of human lymphocyte replication patterns also appeared to support the Ganner and Evans hypothesis [3, 4]. However, in earlier autoradiographic studies, there was evidence that fetal human tissue did not show the same late-replication (LR) sequences as did adult lymphocytes [5, 6]. Moreover, autoradiographic and BrdU replication studies of other tissues in other species also showed variations from direct correspondence between LR and banding patterns [7, 8]. Yet, several studies with the more precise BrdU/stain techniques [9–11] did not clarify the issue of tissue variation in human replication patterns. Willard and others have now confirmed, however, that there are indeed tissue specific differences in human replication sequences [12–14] as well as replication differences in mammalian aneuploid and translocated human chromosomes [15–17]. These differences thus question the Ganner and Evans hypothesis as well as the role of replication sequencing in cellular function.

Utilizing a replication banding technique developed in our laboratory (see MATERIALS AND METHODS), preliminary experiments indeed indicated that some regions in human lymphocyte chromosomes showed negative correspondence between G-bands and LR patterns. Furthermore, these differences also seemed present in reverse order in studies of early-replication (ER) patterns. However, as with all replication studies, the cell-to-cell variation in replication sequences and the general similarity to banding patterns made it difficult to evaluate these differences precisely by simple karyotype comparison. Unlike banding studies, replication analysis involves complex changes in all the chromosomes that are beyond the information processing capability of a human observer. To overcome this problem, quantification of replication analysis was attempted.

The Image Analysis Laboratory of the Tufts-New England Medical Center previously developed computerized techniques for the quantitative measurement of G-banding patterns [18, 19]. Following a period of direct collaboration, we have now been able to modify these techniques for the measurement and comparison of replication patterns. Here we will briefly outline the procedures in the computer processing system and will focus mainly on the cytogenetic results and implications.

Analyses were done on the photographic negatives of ER and LR patterns of human lymphocytes of four normal individuals (figs. 1 and 2). The metaphase spreads analyzed were previously karyotyped, and only examples of the first six autosomes and the X chromosome(s) were selected for this analysis. Using the Tufts automated scanning system, the selected chromosomes were individually digitized with 64 gray levels of discrimination and 0.1 micron picture point spacing. The digitized images were stored on disk for separate analysis. When each chromosome image is recalled, it is magnified on a TV monitor and processed automatically through programmed stages with the possibility of operator intervention. The operator may correct or modify the analysis via a light pen and/or

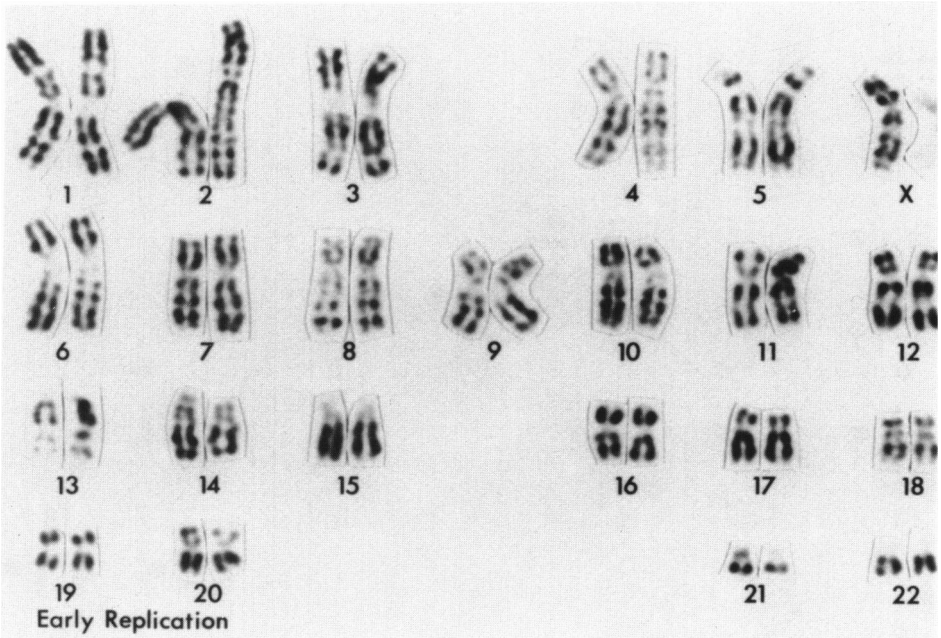

FIG. 1.—Typical HBG-stained ER chromosomes from a normal human female lymphocyte. The ER patterns are the opposite of LR patterns (fig. 2) but not of G-banding.

console instructions. Sequentially, the primary stages of analysis are: (1) chromosome boundary tracing by threshold discrimination; (2) location and marking of centromeres and p and q telomeres; (3) mathematical calculation and tracing of the best fit parabolic or cubic curves to describe the linear axis of each chromatid and the central linear axis of the chromosome (fig. 3); (4) measurement of the summed densities along the linear axis of each chromatid; (5) normalization of the arm lengths of each chromatid to correct for the effects of curvature; and (6) calculation and printout of density profiles for each chromatid and the mean profile for the chromosome (fig. 4). The density curves for the LR patterns were inverted before printout so they could be simultaneously fitted in puzzle fashion with their corresponding ER patterns as well as compared with representative density profiles measured in the same way from trypsin G-banded chromosomes.

MATERIALS AND METHODS

Culturing and Replication Treatments

Lymphocyte chromosomes from normal human donors were prepared by standard whole blood microculture techniques. The cells were grown for a total of 72 hrs under constant rotation at 1/5 rpm in a Lab Line tissue culture rotator. The cultures were set up in Gibco (Grand Island, N.Y.) chromosome 4 media to which 10 μg/ml thymidine (T) and 14.2 μg/ml deoxycytidine (dC) was added to make the cells dependent on salvage T pools and to prevent BrdU or T toxicity. For LR differentiation, the media was removed at 19 hrs before harvest (HBH) by centrifugation and replaced with fresh McCoy's 5A media (Gibco)

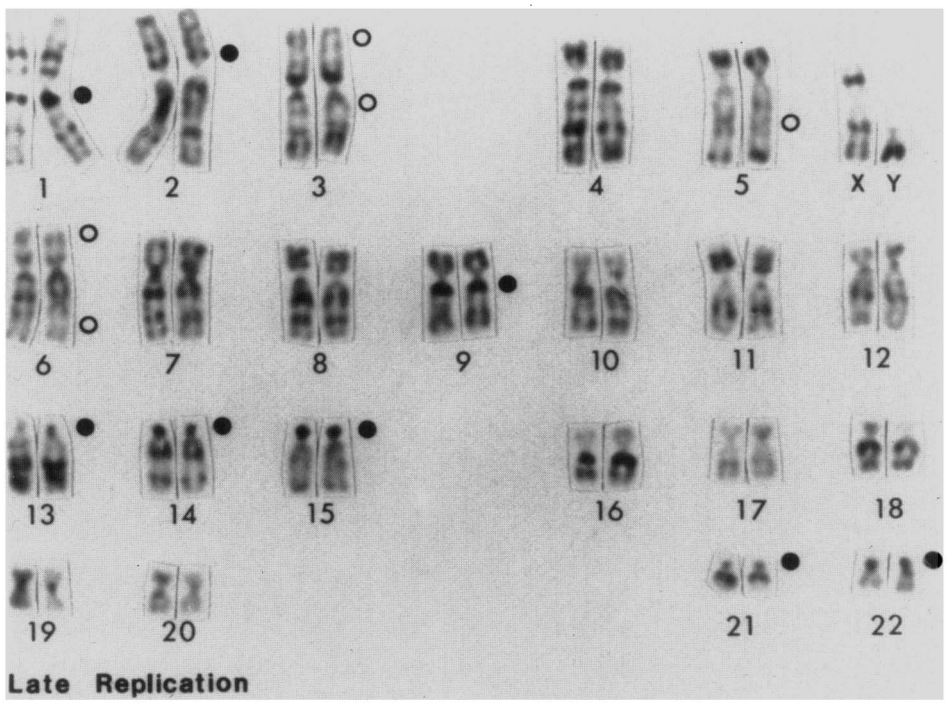

FIG. 2.—Typical HBG-stained LR chromosomes from a normal male. *The symbols* identify the most obvious areas of difference from G-banding. *Solid circles* indicate regions showing dark LR patterns where trypsin G-banding would give light or lighter G-bands. *Open circles* indicate regions with light LR patterns that would otherwise show dark G-bands.

supplemented with 28 μg/ml BrdU, 0.1 μg/ml fluorodeoxyuridine (FdU), 1.5 μg/ml uridine (U), and 13.2 μg/ml dC (Sigma, St. Louis, Mo.). The media were again replaced at 6 HBH with fresh media supplemented with 5 μg/ml T and 6.6 μg/ml dC. Thus, T was incorporated only into the nascent DNA replicating during the final 1–2 hrs of S phase of the last cell cycle. For ER differentiation, the cells were allowed to continue into the last cycle of replication with the initial T and dC supplemented media. At 11 HBH, that media was removed and replaced with fresh McCoy's 5A supplemented with BrdU, FdU, U, and dC as above. Thereby, in the ER cultures, T incorporation in nascent DNA was limited to the first 1–2 hrs of S phase. For mitotic arrest, all cultures were treated with 0.16 μg/ml Colcemid (Gibco) at 1 HBH.

HBG Staining

Replication staining was achieved by the Hoechst-blacklight-Giemsa (HBG) method of Shafer, Madden, and Falek (unpublished observations, 1977). Slides were routinely stained for 10 min with 10^{-4} M Hoechst 33258, rinsed, and covered with several drops of pH 7.1 buffer solution (15 parts 1M Na_2 HPO_4 and 2 parts 0.1 M citric acid) under a loose coverslip. The slides were exposed to 53 J/m^2 per second blacklight (two General Electric 18-inch, 15-watt blacklight tubes at 5 cm above the slides) for 15 min, rinsed in distilled H_2O, immersed in 2 × SSC at 60°C for 5 min, and stained with 2% Giemsa (Fisher, Pittsburgh, Pa.) in a pH 6.8 phosphate buffer for 4.5 min. With good HBG differentiation,

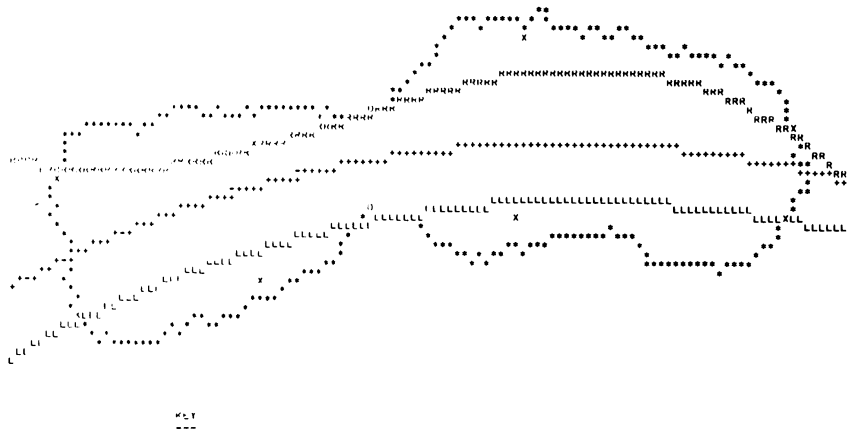

FIG. 3.—Printout of chromosome boundary tracing; automatic determination of centromere and telomere locations; and tracing of the best fit parabolic or cubic curve for the axis of each chromatid.

T-incorporating regions stain dark magenta and BrdU-incorporating regions stain pale blue. Contrast between replication regions can thus be enhanced or decreased by color filter selection.

Image Analysis

The 35-mm photographic negatives of the selected metaphase spreads were analyzed with the Piquant image processing system [20]. Images are digitized with a CRT-driven flying spot scanner and photomultiplier and displayed on a 15-inch console monitor. Both the scanner and monitor are linked to a Nova 840 minicomputer (Data General) equipped with 256K bytes of core memory and extensive peripherals including two 60 megabyte disk drives. The entire process is monitored interactively by a human operator.

For a spread under analysis, each chromosome is tagged by an enclosing rectangle, and its previously determined karyotype assignment is entered by the operator. The chromosome boundary is automatically located by tracing an isodensity contour at a program-determined threshold. The centromere and telomeres are identified by the method of Gallus et al. [21, 22], which is based on local and contextual analysis of the boundary curvature. Axis determination and density measures are based on the methods of Selles et al. [23]. The profiles consist of a series of density readings taken at 0.1 micron intervals along the chromatid axis from the p terminal to the q terminal (see fig. 5). At each interval, the reading is the sum of the optical densities along a line constructed perpendicular to the chromatid axis at that point and extending from the central axis to the outer boundary of the chromosome. Each perpendicular is also stepped off laterally in 0.1 micron intervals from the chromatid axis. Actual density readings are made at each of these precise sampling points by interpolating from the densities of the four surrounding pixels in the quantized raster.

Since two chromatid profiles are obtained for each chromosome, they may be separately analyzed as paired replicates, or added together to form a profile for the chromosome. In the latter case, the chromatid profiles are first aligned at the centromere; then for each arm, the shorter profile is adjusted to match the longer profile in length. Alternatively, profiles may be normalized to standard arm lengths for the chromosome. A typical chromosome is

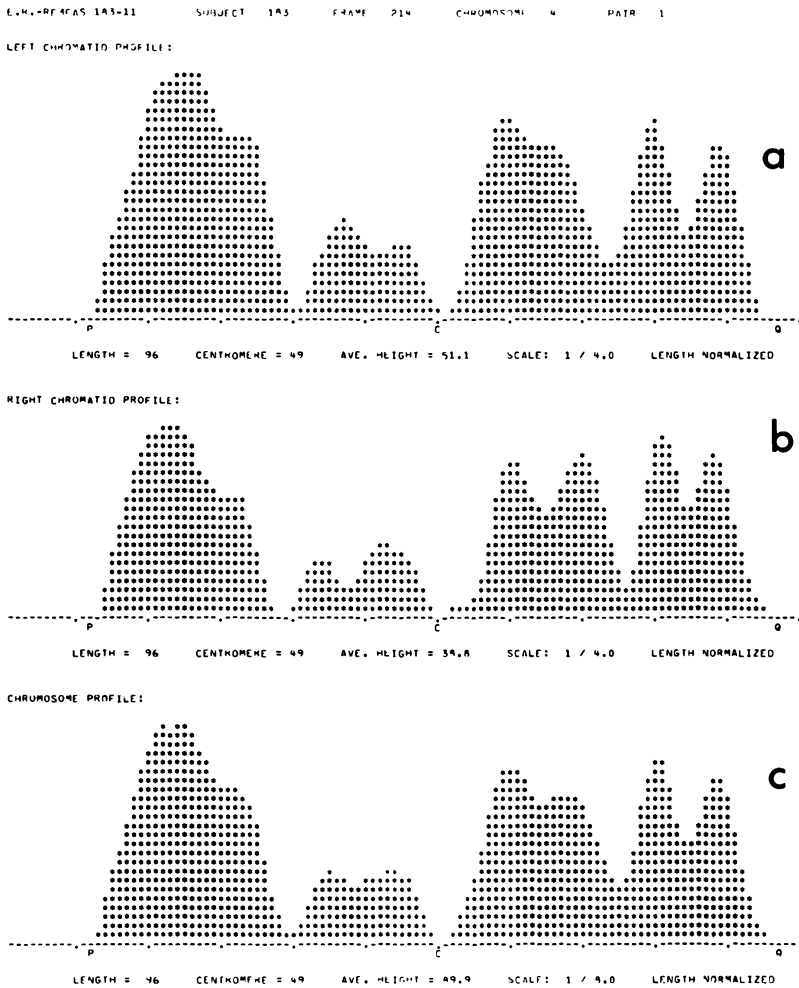

FIG. 4.—Printout of density profiles of ER patterns of a human lymphocyte chromosome 1. *a*, Left chromatid summed densities; *b*, right chromatid summed densities; *c*, mean density profile of both chromatids (lengths normalized).

processed in the above manner in about 15 seconds with about 80% of the chromosomes requiring no intervention.

Photography and Image Selection

Metaphase spreads were photographed with a Zeiss Universal Photomicroscope using a green interference filter (520–560 nm), 35-mm Kodak SO-115 photomicrography film, and Kodak D-19 developer. Low-to-moderate contrast negatives were prepared to match the optical density range of the scanner (0–2 OD U). The scanning system requires much less contrast than that required for visual discrimination. Negatives were selected for analysis that had a minimum number of overlapping chromosomes as the density patterns of such

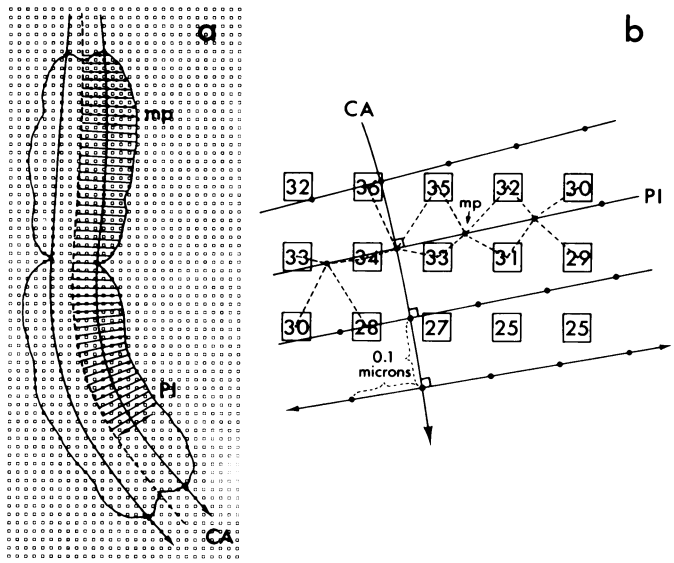

FIG. 5.—Representation of density measurement system. *a*, Once the chromatid axis (*CA*) and the central axis are determined, perpendicular intercepts (*PI*) are defined at each 0.1 micron interval along the *CA*; measurement points (*mp*) are then stepped off at 0.1 micron intervals in either direction along the *PI* from the *CA* to the *central axis* and to the *outer chromosome boundary*. *Open squares* graphically represent the raster of pixels on the monitor but not the true, higher resolution obtained. *Solid dots* represent the location of measurement points. *b*, Detail of measurement system; each *mp* is translated into a single density value by interpolating the gray level density values of the surrounding four pixels based on the relative position of the *mp* to each pixel. At the *mp* indicated by *arrow*, the *mp* value will be skewed toward the pixel gray levels that are closer (*33, 31*); *mp* values for each *PI* are summed.

chromosomes cannot be accurately quantified. Perfect images were not, however, required, as touching chromosomes could be easily separated and the density measurement system could accurately follow curved or S-shaped chromosomes.

RESULTS

Replication patterns for both early and late S showed consistency from cell to cell and from individual to individual in terms of the specific chromosomal locations of regions undergoing replication in each chromosome. The replication patterns of each chromosome differ, however, in the relative degree or intensity of replication occurring in specific replication "bands." Moreover, the location and intensity of a number of LR "bands" do not correlate with the G-band patterns for those chromosomes. Figure 6 shows tracings of chromosome 1 ER patterns from several cells and individuals. All these chromosomes show similar patterns and nine major ER peak locations. Figure 7 shows a similar set of tracings from chromosome 3 ER patterns that have five major peak locations. In both sets of tracings, particular corresponding peak locations show variation in peak height that reflects differences in the relative intensity of replication occurring in that region. It is not yet determined whether these peak variations demonstrate specific differences in replication intensity that occur between individuals or between cells.

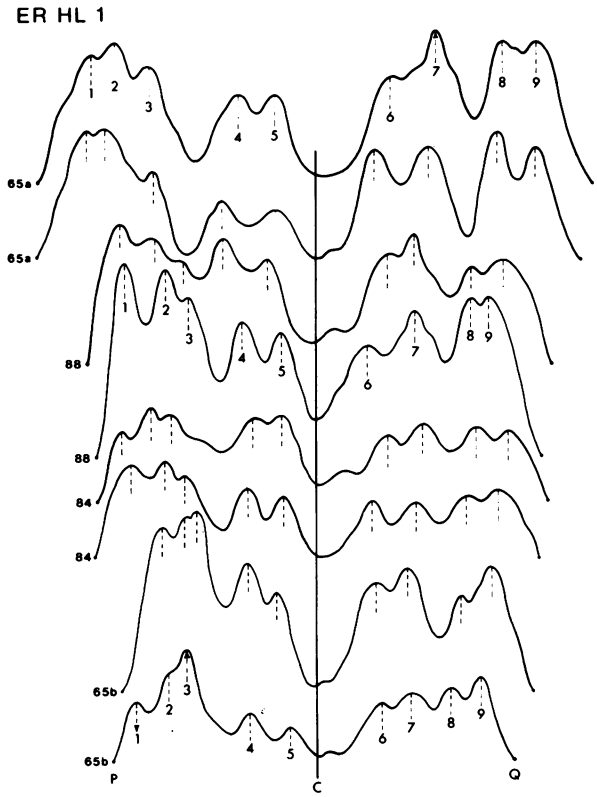

FIG. 6.—Density profiles of several chromosome 1 ER patterns from different individuals. They were arranged vertically according to relative length, not pattern. *Matching nos.* indicate homologous chromosomes from the same cell of the same individual. All no. 1 chromosomes show nine major ER peaks in the same relative locations along the chromosome. Yet, some peaks differ in relative intensity (height).

However, the consistency both in peak location and in the relative height of most other peaks indicates that these specific variations are not the product of random unrelated staining effects, but, rather, they reflect true intercellular and/or inter-individual replication differences.

Figures 8 and 9 graphically compare the sum density curves for all ER, LR, and G-band patterns studied. For this initial analysis, these curves were achieved by superimposing individual tracing to derive a generalized ER, LR, or G-band curve for each chromosome. For future analyses, the existing capability of the Piquant system for carrying out this calculation will be utilized. The alternate dotted line segments indicate regions where one or more individual curves differed significantly from the general curve. These variations are, however, in band density, not in location. In general, the ER patterns are the opposite of the LR patterns except at the centromere where both are structurally constricted. On the other hand, although most of the LR patterns are similar to the G-band patterns as reported in

previous studies [1, 3, 4], some regions are distinctly different. Moreover, these differences from G-banding are confirmed in reverse form in the ER patterns.

Chromosomes 1, 4, and the active X show the most correlation between the LR and G-band patterns. However, there are regions of limited inconsistency. In the various ER patterns of chromosome 1 (fig. 6), the p terminal group of three adjacent ER bands is not always the most intense ER region of the chromosome even though the corresponding region by G-banding is consistently pale. Moreover, the prominent LR region just below the centromere of chromosome 1 shows more replication intensity than the corresponding G-band staining effect would suggest. This latter difference is also consistent with the direct visual observation that the C-band regions of chromosome 9 as well as of 16 are also intensely late-replicating even though the chromosome 9 C-band region is both G- and Q-band negative (fig. 2). Chromosomes 2 and 5 show less similarity between LR and G-band patterns but these differences are limited. The most consistent difference in chromosome 2 is the relative intensity of the major LR band just above the centromere. Chromosome 5 differs largely in the ER and LR bands just below the centromere.

The most divergence between the replication patterns and G-bands occurs in chromosomes 3, 6, and the inactive X. In chromosome 3, the upper and lower arms have nearly identical mirror image G-patterns, whereas the ER and LR

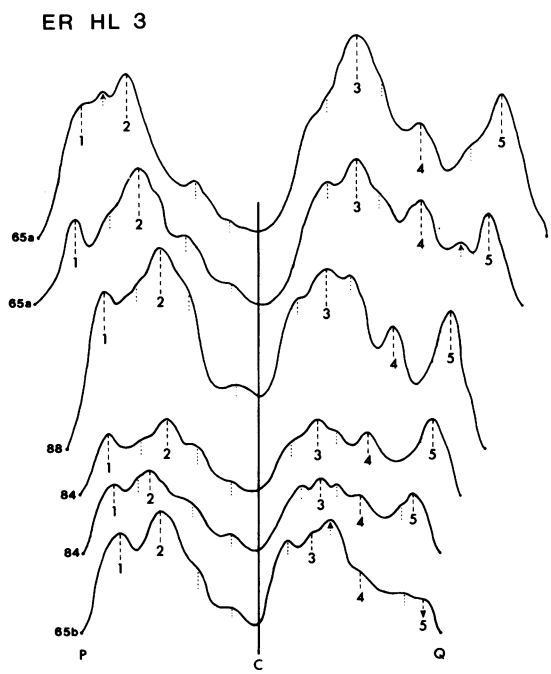

FIG. 7.—Density profiles of several chromosome 3 ER patterns from different individuals. They show five major ER peaks and several minor variant peaks.

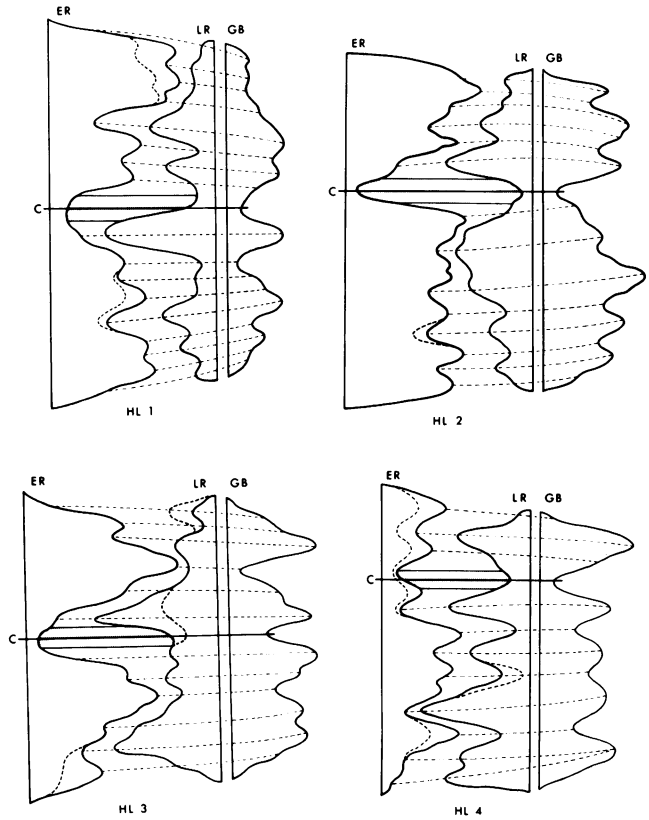

FIG. 8.—Combined, generalized density profiles of *ER*, *LR*, and G-banding (*GB*) from human lymphocyte (*HL*) chromosomes 1–4. *Solid profiles* show the major patterns; minor variants are indicated by *broken line segments. Thin dashed lines running horizontally across the profiles* trace the matching peaks and valleys that might otherwise be misread because of the progressive chromosome condensation of *LR* over *ER* patterns and *GB* over *LR* patterns. *ER* and *LR* patterns generally fit together except for the indicated centromere gap area. *LR* and *GB* patterns match fairly well in *HL 1* and *4*, show less similarity in *HL 2*, and are distinctly different in *HL 3*.

patterns of this chromosome both diverge in a consistently alternate pattern in both arms. In chromosome 6, the prominent terminal G-band of the upper arm is early-replicating, not late, and the G-band positive regions of the distal half of the lower arm are also early-replicating. The inactive X is particularly interesting in that the few regions that are early-replicating are a selective subset of the ER patterns of the active X rather than a unique replication pattern.

Density patterns from various individuals that best represent the generalized ER and LR patterns of figures 6 and 7 are shown in figure 10. More accurate representative curves can be generated directly by the Piquant system by averaging the locations, widths, and heights of a much larger sample of individual profiles. Evaluations of chromosomes from specific individuals or tissues could then be

made by comparing the location, size, and/or intensity of replication sequences to a set of replication templates.

DISCUSSION

The image analysis of chromosome replication patterns presented here demonstrates the possibility of quantifying ER and LR patterns in a manner that allows comparative evaluation. With visual methods, such analyses were previously limited to comparing the patterns of a single chromosome [12, 15]. With further refinements of the image processing system, it will be possible to quantitate and compare the replication patterns of all the chromosomes in different tissues or individuals and perhaps even in different populations.

Currently, banding pattern analysis with G, Q, R, C, or other banding methods are extensively used for both clinical diagnosis and evolutionary comparisons. Yet, these uses are limited in that banding reveals only the location of visually similar chromatin segments, not the function or genetic activity of such segments. On the other hand, it is well known that the inactivated X chromosome in mammalian females is late-replicating. More recently, Latt et al. [15] have shown with the BrdU technique that in individuals with supernumerary X chromosomes, these

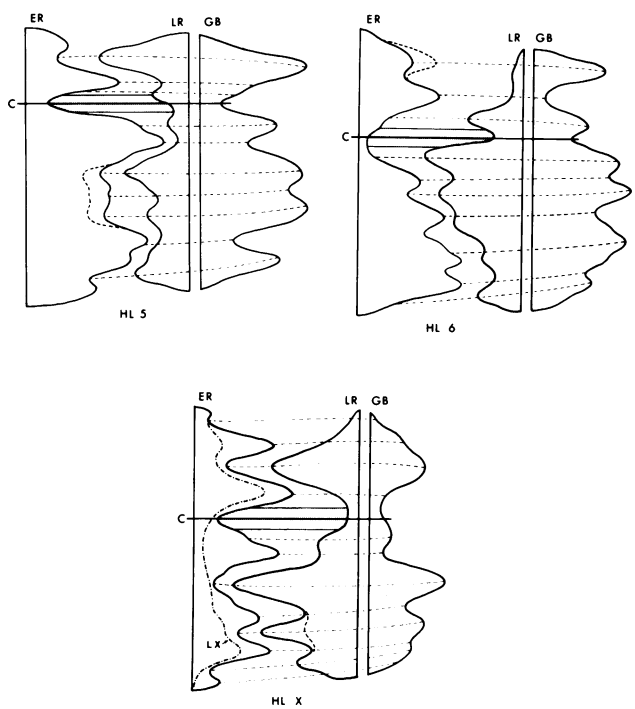

FIG. 9.—The *LR* and G-banding patterns of chromosomes *HL 5* and *6* show several different regions. The *LR* and *GB* patterns of the active X chromosomes of males and females are quite similar. While the inactive (late-replicating) X (*LX dot-dash line*) of females differs from the active X, the *LX ER* peaks that do occur are in the same location as the *ER* peaks of the active X.

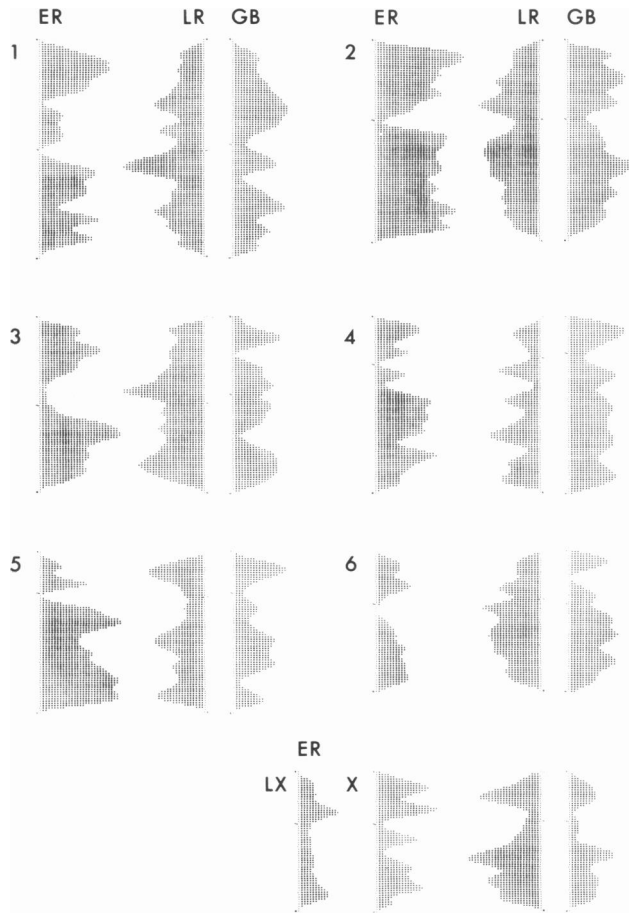

FIG. 10.—Selected actual density profiles from various individuals that best represent the generalized profiles shown in figures 6 and 7.

chromosomes are also late-replicating and presumably inactivated. Willard's [12] findings that replication patterns vary in different tissues also links replication sequencing to differential gene activation. Couturier et al. [17] have strongly confirmed this association by demonstrating measurable differences in autosomal gene activity in a family with X translocated 21 chromosomes. They showed that the levels of superoxide dismutase (a chromosome 21 gene) correlated with the different degrees of LR of the 21 chromosomes in each individual.

The findings presented here quantitatively demonstrate that there is not a one-to-one correspondence between late-replicating, presumably A-T-rich DNA regions and the G- or Q-positive bands in human lymphocytes. This means that replication sequencing may be related to, but is not determined by, band organization. Previously, one of us (D. A. S.) proposed a hypothesis by which gene function and cellular differentiation may be linked to replication sequencing [24]. This

thesis will be presented in detail elsewhere. In essence, the hypothesis suggests that gene activation depends on ER that allows selective binding of early S nonhistone proteins. In differentiating tissue, nonfunctional or reduced function gene regions progressively shift into LR where they are bound with late S nonhistone proteins that "heterochromatinize" and repress transcription. In this view, selective "heterochromatinization" (i.e., localized molecular condensation and inactivation of structural genes) during differentiation is facilitated by the degree and extent of repetitive or intron DNA that is interspersed within or adjacent to structural gene regions. Therefore, in highly differentiated tissues such as T-lymphocytes, considerable correspondence would be expected between G-bands and LR patterns. In such tissues, most of the structural genes in the positive G-bands would be inactivated since these regions contain a high proportion of intermediate repetitive DNA [25–27]. On the other hand, less differentiated tissues such as fibroblasts should show more early- to mid-replication sequences in G-band regions because of multiple generalized functions. Such tissue specific contrasts would not be expected in the negative G-bands (interbands). These regions would always be early replicating as they primarily contain structural genes with little or no interspersed repetitive DNA and presumably provide functions essential to all cells.

While such a radical model will require considerable support, critical evidence is accumulating. Schmidt [28] has recently shown that there is a distinct gap between the ER and LR phases in human lymphocytes. This finding is also supported by the evidence presented here. The ER and LR curves of figures 6 and 7 fit together despite the 5-hr gap between the end of the ER pulse and the beginning of the LR pulse. This bimodal S phase is indeed generalized for diploid cell lines as contrasted with transformed cell lines [16] that have a short, unimodal S phase like undifferentiated cells do. Moreover, it is generally known that as cell differentiation occurs in vivo and in vitro it is accompanied by a slowdown or a cessation or replication. These phenomena are consistent with the model.

If Shafer's hypothesis is correct, replication analysis takes on a unique significance as a measure of differential genetic activity. Replication pattern analysis is not just another banding procedure, but constitutes a functional rather than a descriptive approach for cytogenetics. In such a context, the further development and application of quantitative cytogenetic research tools such as described here would be both important and imperative.

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