QUANTITATIVE TRITIUM AUTORADIOGRAPHY
OF MAMMALIAN CHROMOSOMES

I. The Basic Method

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ABSTRACT
A technique has been developed that allows repeated autoradiographs to be made of the isotope distribution in the chromosomes of a single cell. A series of 10 separate autoradiographs were made of a Chinese hamster diploid male metaphase cell which had been labeled with tritiated thymidine during the first 15 minutes of its DNA synthesis period in the previous interphase. Each autoradiograph had low grain densities above the chromosomes so that quantitation was feasible. The separate autoradiographs were photographically combined into a single composite in which grain images were converted to lines oriented at right angles to the chromosome axis. The line densities were then measured with a recording microdensitometer to yield graphs reflecting the isotope distribution along each chromosome. The area under each graph was directly proportional to the total number of grains counted above the corresponding chromosome in the 10 separate autoradiographs. The distribution of isotope along the chromosomes was different for each chromosome, and in some cases homologs also differed in their early labeling patterns.

Tritium autoradiography has proved to be an extremely valuable tool in cytogenetics. The basic concept of asynchronous chromosome duplication (1, 2), with the subsequent elucidation of specific duplication sequences in the chromosomes of certain mammals (3-5), has been a direct result of the application of the method of autoradiography. In many species, autoradiography provides the best method for sex chromosome identification (4, 6). Chromosome autoradiography has been largely qualitative, however, and its usefulness could be greatly improved if accurate quantitation were possible.

Quantitation should be possible, since the production of developable silver grains in a photographic emulsion is proportional to the amount of radiation to which it is exposed. Thus, under ideal conditions, one may count the number of silver grains over a particular structure in an autoradiograph and assume that the total number of grains reflects the amount of isotope contained in the structure. In comparing two such structures, numerous variables, such as distance from source to emulsion, composition of intermediate media, uniformity of background, etc., must be carefully monitored.

In counting silver grains on mammalian chromosome autoradiographs, several additional factors become important. Mammalian chromosomes are not large structures (approximately 1 \( \mu \) in width and generally not more than 10 \( \mu \) in length), so it is important to make the grain size as small as possible. Even if the grain size approaches the limit of resolution of the light microscope (0.2 \( \mu \)), it is not possible to accumulate very many individual grains over a chromosome before.
coincidence and overlapping limit the accuracy of quantitation. Thus, if grain densities are low enough to make counting possible, the total number of grains over a chromosome is not sufficient to be statistically reliable.

The obvious way to circumvent such a limitation is to take more than one sample of the grain distribution along a chromosome. Two approaches are possible. One method is to group the data obtained for a given chromosome as it occurs in many different cells of a culture subjected to a given treatment. Stubblefield and Mueller (2) used this approach in demonstrating early and late replicating segments of chromosome 2 of HeLa cells. It must be assumed, however, that all the cells behave alike and that the investigator can accurately recognize the chromosome in each cell. Since most chromosomes occur at least twice in mammalian cells, a further assumption is that homologs behave in a similar manner. This, however, appears not to be the case in studies of X chromosomes of some mammalian females, where one X chromosome is duplicated earlier than the other (4). A second method is to make a series of autoradiographs of the chromosomes of a single cell. This tack eliminates the problems described above, but it introduces difficulties of a different nature.

Autoradiographs prepared in the usual manner with either stripping film or liquid emulsion are essentially permanent preparations. The emulsion adheres tightly to the cell preparation and cannot be removed by peeling without also destroying or at least damaging the underlying cells. It is possible to dissolve the emulsion in hot water or in solutions of high or low pH, but such methods usually leave the silver grains still firmly attached to the cells. Chemical oxidation of the silver is feasible, but such harsh treatment may remove some of the isotope. Chromosomes, in particular, are visibly altered in appearance by these techniques.

The solution to this problem was achieved by overlaying the cell preparation with a very thin, but strong, plastic film. Formvar is excellent for this purpose (7, 8). Bishop and Bishop (9) independently developed a similar technique using Vinalak, but they encountered difficulties in keeping the stripping film attached to the slide. Plaut (personal communication) found that collodion also worked satisfactorily. The plastic film provides a smooth surface from which the emulsion can be easily peeled without disturbing the underlying cells, yet it is not thick enough to absorb an appreciable amount of radiation or seriously affect resolution.

Using the Formvar film method, a series of autoradiographs was obtained from a single cell. The way in which the data were assembled and analyzed is the main subject of this report.

METHODS AND RESULTS

Cell Culture

For this study a diploid Chinese hamster male cell line designated “Don” (3) was used. Cells were grown in McCoy’s medium 5a, supplemented with 20 per cent fetal calf serum. A logarithmically growing monolayer culture in a T-30 flask, was treated with 0.1 μg/ml 5-fluorodeoxyuridine (FUdR) for 12 hours. Tritiated thymidine (specific activity, 6.7 c/mmolc) was then added (1.0 μc/ml) for 15 minutes; the medium was then changed, and conditioned medium containing unlabeled thymidine (1.0 μg/ml) was introduced. The cells were grown for an additional 5 hours; then Colcemid (0.06 μg/ml) was added to the medium for 4 more hours of incubation. Cells were harvested by trypsinization and were subsequently treated with 1 per cent sodium citrate solution for 10 minutes at room temperature to spread the chromosomes of metaphase cells. The culture was fixed with 50 per cent acetic acid for 30 minutes at room temperature. Squash preparations were made using unstained cells, and the coverslips were temporarily sealed to the slide with Kroenig’s resin. The majority of cells in the culture were synchronized by the FUdR treatment, so that the tritiated thymidine was incorporated into the DNA synthesized during the first 15 minutes of the synthesis period in each cell. In this way many cells in the same stage of synthesis could be compared on a single slide. In this report we shall confine our attention to a single cell for demonstration purposes. Comparative data on different cells will appear in a later communication.

Photomicrography

The cell chosen for this report is shown in Fig. 1. The photomicrograph was made using a Zeiss microscope with an oil immersion 100× dark phase contrast objective and a Leitz Aristophot camera with a 6× Periplan ocular in the monocu-
Figure 1. Metaphase chromosomes of the male diploid Chinese hamster cell. Phase contrast. Magnification is indicated by scale on Fig. 3.

Figure 2. Identification of the chromosomes in Fig. 1. Chromosomes 7 and 8, also 10 and 11, cannot be morphologically distinguished, but here they are arbitrarily paired to simplify the presentation.

Figure 3. Autoradiograph VIII of the chromosomes in Fig. 1. Film exposure was 65 hours. The cell was labeled with tritiated thymidine during the first 15 minutes of the previous period of DNA synthesis. Bright field, no stain.

Figure 4. Negative image of perforated film. The grain images in Fig. 3 (on transparent film) were drilled out with a 0.5-mm high-speed drill, and the film was stained with crocein scarlet. This procedure converts all grains to uniform pinholes.
lar tube. Ansco Super Hypan film (4 × 5 inches) was used; it was developed in Kodak DK-60a developer at 68°F for 5 minutes, rinsed in dilute acetic acid, and cleared in Edwal Quick-Fix for 3 minutes.

In Fig. 2 the chromosomes of this particular cell are numbered (10). All the chromosomes of this species are morphologically identifiable with the exception of chromosomes 7 and 8 and chromosomes 10 and 11. Partly on the basis of labeling patterns (to be described later in this report), these 8 chromosomes have been assigned the numbers shown. The X chromosome can be distinguished from chromosomes 4 by the secondary constriction in the long arms.

Preparation of Autoradiographs

After all the favorable cells had been located and photographed, the preparation was frozen on solid carbon dioxide, the sealing resin was scraped off, and the coverslip was quickly flipped off with a sharp scalpel. The slide was washed in two changes of absolute ethanol and air dried.

The Formvar film was prepared from a 0.5 per cent solution of the powder in ethylene dichloride; 20 ml of the solution was placed in a 100-ml graduated cylinder. A clean slide attached to a string with a paper clip was lowered halfway into the solution. The slide was then raised above the level of the liquid and drained for 60 seconds in the saturated vapor of the cylinder. The slide was then removed from the cylinder; the ethylene dichloride evaporated immediately, leaving a very thin film of Formvar on the slide. The slide was scraped at the edges with a scalpel to free the edge of the film. The area of the slide covered by the film was then determined by condensing moisture from the breath on the slide; this also served to loosen the film from the slide. Upon gently lowering the slide into a water bath (room temperature) at an angle of 45°, the film could be seen by reflected light to float free on the water surface. The film was then picked up on the slide bearing the chromosome squash preparation and set aside to dry.

Autoradiographs were prepared by the method of Schmid and Carnes (11) using Kodak AR-10 stripping film. The scored glass plate bearing the stripping film was immersed in 70 per cent ethanol for 1 minute to partially hydrate the emulsion and was then transferred to absolute ethanol. The individual squares of emulsion were peeled off under the ethanol to prevent any background exposure from static electricity. The emulsion was stiff and could be handled conveniently with forceps. The emulsion was inverted and floated on the surface of a water bath to hydrate the film. The hydrated film was picked up on the Formvar coated specimen slide, immersed in water for 2 minutes to complete the hydration process, and finally smoothed down with a camel's-hair brush. The slides were dried slowly in a special light-proof box (11) and then stored in a dry nitrogen atmosphere in sealed plastic slide boxes at 4°C.

After an appropriate exposure period (approximately 72 hours), the autoradiographs were developed in Kodak D-19b developer (68°F) for 2 minutes, rinsed in water, and fixed for 2 minutes in Kodak Acid Fixer. The slides were washed in running water for 2 minutes and then air dried. A coverslip was mounted on the slide with glycerol, and two corners of the coverslip were secured with Kroenig’s resin.

The autoradiographs of the cells located earlier were then found and photographed. The photomicrographs of the autoradiographs were made on Ansco Super Hypan film at exactly the same magnification as the chromosome photographs. A bright field condenser was used, so that the silver grains appeared small and black, and the chromosomes were only faintly visible in the negative (Fig. 3).

After all the autoradiographs had been photographed, the Kroenig’s resin was removed from the slide with a scalpel, and the slide was immersed in water. The coverslip could soon be teased away from the slide and the glycerol extracted in several changes of water. With the emulsion still moist, a corner of the film was loosened, and the autoradiograph was peeled from the slide with forceps. The emulsion separated cleanly from the Formvar film, which remained firmly attached to the cells. The slide was now ready for new stripping film to be applied as described above. This cycle was repeated 9 times to yield a total of 10 consecutive autoradiographs, each of approximately 3-day exposure. A final autoradiograph was then prepared with an exposure of 31 days.

Photographic Integration of the Autoradiographs

At this point in the analysis the data were recorded as silver grain images in the photographic negatives of the 10 autoradiographs. The photomicrographs were all taken at exactly the same
TABLE I

Distribution of Silver Grains over the Chromosomes in the 10 Separate Autoradiographs

<table>
<thead>
<tr>
<th>Chromosome no.</th>
<th>Autoradiograph no.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
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<td>26</td>
<td>31</td>
</tr>
<tr>
<td>(3) X</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>(3) Y</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
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</tr>
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<td>10-11</td>
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</tr>
<tr>
<td>Total</td>
<td>263</td>
<td>277</td>
</tr>
<tr>
<td>Background not over chromosomes</td>
<td>15</td>
<td>55</td>
</tr>
<tr>
<td>Exposure time (hours)</td>
<td>76</td>
<td>68</td>
</tr>
</tbody>
</table>

magnification and could be accurately superimposed by using the faint chromosome images as cue marks. However, the silver grain images were not uniform; some grains were darker than others, probably because of slight variations in vertical position with reference to the focal plane of the microscope objective. In addition, the grains were of varying sizes and shapes, and some of these latter variations were undoubtedly the result of multiple activation of a grain (coincidence radiation). In order to eliminate the variations, the following procedure was devised.

A positive image of the original negative of each autoradiograph was made on Kodak Kodalith (Super Ortho) film (chosen for its thin film base and high contrast). This film was developed for 3 minutes in Kodak DK-60a at 68°F. In the finished transparency, the image of each silver grain was drilled through with a high speed drill bit 0.5 mm in diameter. This converted each image to a pinhole of uniform diameter. As the perforations were made, records were kept of the total number of grains found over each chromosome in each autoradiograph (Table I). The interpretation of the grain count of the autoradiograph in Fig. 3 is shown in Fig. 4. A few of the very weak grain images were overlooked, and the multiple grains were interpreted arbitrarily, but any mistakes were assumed to be random.

The perforated films were carefully superimposed on a photograph of the chromosomes and all were trimmed on a paper cutter to a common corner. The films were stained with a 1 per cent solution of Kodak crocein scarlet to render the background photographically opaque. Finally, all perforations were carefully brushed clean to eliminate any debris from the earlier drilling. Fig. 4 was obtained by placing the stained per-
forated film in an enlarger and printing its negative image on photographic paper.

The size of the grains could be substantially reduced by using each pinhole in the perforated film as a lens to project the image of a "point" source of light. The effect can be seen in comparing Fig. 5 with Fig. 4. A Beseler 45MCR enlarger with a 250-watt bulb was used. The perforated films were mounted 9 cm above the enlarger table. The point source consisted of a 50-mm f/4.5 lens stopped down to f/22 mounted on the enlarger at a distance of 90 cm from the table. Consecutive projection using the entire sequence of 10 autoradiograph patterns resulted in the composite photograph shown in Fig. 6. The perforated films were all carefully oriented, using the common edges as a guide, so that the resulting superposition would be accurate.

A brief examination of Fig. 6 revealed several interesting facts about the distribution of the isotope in the cell. The background radiation appeared to be uniformly distributed and was relatively light, considering that it represented all the background accumulated in 10 separate autoradiographs. All chromosomes incorporated at least some isotope during the first 15 minutes of DNA synthesis; however, chromosomes X, Y, 10, and 11 appeared to contain considerably less isotope than the others (see monitor, Fig. 2). As expected, daughter chromatids appeared to be equally labeled. However, along the major axis of most chromosomes there appeared to be considerable variation in radioactive intensity from region to region.

**Conversion of Grains to Lines**

Since the genetic elements are probably arranged in linear sequence, the distribution of isotope along the long axis of the chromosome is of major interest. Daughter elements are presumably identical, so it would be advantageous to eliminate the lateral dimension and add together all the silver grains occurring at each locus along a chromosome. This was accomplished as described below.

The chromosomes in Fig. 1 were divided into groups having parallel orientation; one such group consisted of chromosomes 2, 8, 9, and 10, which occupy the areas shaded in Fig. 5. A mask of black paper was then prepared in which the shaded areas of Fig. 5 were cut out. When the mask was placed over a perforated film, all the holes not occurring in the areas occupied by these chromosomes were covered. Eight different masks were required to include all the chromosome axes occurring in this particular cell. No two chromosome areas were allowed to overlap.

Next, each grain position was converted into a thin line at right angles to the long axis of the chromosome. This was accomplished by using the pinhole camera effect as in Fig. 5, but instead of a point source, a linear light source was projected through each pinhole. The linear light source consisted of a slit 0.8 × 57 mm cut in a sheet of opaque paper; it was mounted in the film holder of the enlarger, which could be rotated 180°. The enlarger lens was removed. The distance from the slit to the enlarger table was 99 cm, and the perforated film was again mounted 9 cm above the table. The resulting projected line was about twice the width of the chromosomes. Using the perforated film shown in Fig. 4, the pattern seen in Fig. 7 was produced by sequentially projecting the linear light source through the pinholes exposed by each of the eight masks, one at a time, with the linear source oriented at right angles to the chromosome axis in each case.

A more useful result was obtained by using a single mask and sequentially changing the perforated films. The composite result, using all 10 perforated films (representing the 10 separate autoradiographs) and the mask for the 4 chromosomes shaded in Fig. 5, is shown in Fig. 8. The density of the lines along each chromosome axis was proportional to the total number of silver grains which occurred at that locus in all 10 autoradiographs. Such line patterns were prepared for the chromosomes occurring in each of the eight different orientations on Kodak Gravure Copy film. The film was developed for 2 minutes at 68°F in Kodak DK-60a developer.

**Densitometer Tracing**

Finally, a graph of the line density along each chromosome was prepared by tracing through the center of each line pattern on a Joyce-Loebl double beam recording microdensitometer (Model E12 MKIIIB). The resulting graphs are shown in Fig. 9. The original graphs were made on paper weighing 5.0 mg/cm². The area under each graph was measured by simply weighing the excised graph. In Fig. 10, the computed area (in cm²) for the graph of each chromosome is plotted against the total number of silver grains (Table I) which
**Figure 5** Pinhole projection of point source of light using the perforated film used to make Fig. 4 (see text). Grain diameters are somewhat reduced by this procedure. Magnification is indicated by scale on Fig. 7.

**Figure 6** Composite combining the perforated films of all 10 autoradiographs projected as in Fig. 5.

**Figure 7** Pinhole projection of a linear source of light using the perforated film used to make Fig. 4. Each group of parallel chromosomes (shaded areas of Fig. 5 represent one group) was printed separately with the linear light source oriented at right angles to the chromosome axis.

**Figure 8** Pinhole projection of a linear source of light using the pinholes falling in the shaded areas of Fig. 5 (one set of parallel chromosomes) on the perforated films from all 10 separate autoradiographs.
DISCUSSION

It should be emphasized that since the procedure presented in this report is a refinement of existing methods, all the precautions normally necessary for autoradiography in general also apply in this method. It is of primary importance that the chromosomes all lie in the same plane and that there be no overlying cytoplasm. The cells must be squashed so flat that the chromosomes contact both the slide and the coverslip. The chromosomes must also be well spread so that there is no overlapping.

For quantitative studies of tritiated thymidine incorporation, it is also important to inhibit endogenous thymidine synthesis. Either FUdR or Amethopterin can be used (12).

The use of AR-10 stripping film seems preferable to the liquid emulsion technique, in that the film can be more easily removed after exposure.

A careful analysis of the data in Table I reveals several interesting facts. Apparently, repeated processing of the autoradiographs caused no loss of isotope from either the cell as a whole or the individual chromosomes. Background was quite variable from one autoradiograph to the next, possibly as a result of handling and storage of the film rather than of cytoplasmic incorporation of the isotope. When allowance is made for background variation, the total number of grains counted over all chromosomes appears to be linearly related to the exposure time, as one would expect.

In making the line images for densitometer tracing, it is important to use a continuous tone film, such as Kodak Gravure Copy film, and to use low exposures so that the film is not saturated, or else quantitation will not be reliable.

A study of the isotope distribution patterns in Fig. 9 reveals significant differences between homologous chromosomes in chromosomes 2, 5, and 6 (arrows). In each case a distinct peak in one homolog appears to be almost entirely absent in the other. Chromosomes 7 and 8, which are morphologically indistinguishable, had four different patterns of initial replication; these are arbitrarily paired in Fig. 9 as the two with the most and the two with the least amount of isotope labeling. Which pairs are actually homologous is not known. The same problem exists for chromosomes 10 and 11; however, in this experiment not enough numbers of grains were found on these chromosomes to establish any significant differences.

It has been clearly established that in many female mammals the two X chromosomes are markedly different in the time of their DNA replication (4). In the usual case, one X-chromosome is replicated late in the period of DNA synthesis, whereas part or all of the homolog is replicated earlier. It now appears that this sort of homolog asynchrony may be characteristic of all chromosomes, and that the sex chromosomes display an extreme case that is easier to resolve.

The minor differences between homologous chromosomes in the graphs of Fig. 9 are not significant. Thus, the homologous short arms of chromosome 2 appear to be identical within the limits of resolution of this method. However, the major fluctuations in isotope distribution along the chromosomes are real. Each major peak represents the position of a large number of DNA molecules at a given locus that begins replication at the start of the DNA synthesis period of the cell. Those regions containing relatively less isotope are rich in DNA molecules that do not begin replication until later. Thus, the earlier conclusion of Stubblefield and Mueller (2) that DNA synthesis is a focalized phenomenon along the mammalian chromosome is supported. The regulation of the timing of DNA synthesis in each site appears to be determined by the molecular architecture of the interphase chromosome at that site.

Although chromosomes 10, 11, Y, and the long arms of chromosome X are largely duplicated late in the DNA synthesis period (3), these regions all incorporate a significant amount of isotope during the first 15 minutes of synthesis. Earlier work on the morphological identification of early and late replicating sites in the chromosomes of this same cell strain (13) indicates that some early replicating segments do indeed exist in these chromosomes.

A 743-hour autoradiograph of the cell in Fig. 1 is presented in Fig. 12. A point by point comparison (arrows) with Fig. 11 (composite autoradiograph, same as Fig. 6) shows that most of the radioactivity peaks visible in the “synthetic” composition are also apparent in the long exposure autoradiograph. This suggests that although the individual grains cannot be counted in Fig. 12, the information is nonetheless present in the photograph. Coinci-
Figure 9  Densitometer graphs compared with the line figures (as in Fig. 8), the composite autoradiographs (Fig. 6), and the chromosomes (Fig. 1). The arrows designate regions where homologous chromosomes are markedly different in their early labeling capacity. The vertical scale (upper right) indicates the approximate number of grains coinciding at each locus of chromosome length (approximately 0.1 μ).
Figure 10 Comparison of the areas under the graphs (Fig. 9) with the total number of grains in the 10 separate autoradiographs (Table I) for each chromosome. The linear relationship between these two functions indicates that the graphs in Fig. 9 are quantitatively accurate (average error is ±9 grains).

Figure 11 Composite autoradiograph (same as Fig. 6). Compare with Fig. 12. Magnification is indicated by the scale on Fig. 12.

Figure 12 Autoradiograph of cell exposed for 743 hours. Arrows indicate points of comparison with Fig. 11. Individual grains cannot be counted, but the similarity of labeling pattern in the two figures is apparent.

dence grains appear to be bigger and blacker in proportion to the radiation intensity. Experiments are currently in progress to find empirically the proper combination of film characteristics that will permit the quantitative recovery of the information from single autoradiographs where grain densities are too high for direct counting. The advantages of such a short-cut method over the
more detailed procedure presented in this report are readily apparent.

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