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A.V. Carrano, J.W. Gray and M.A. Van Dilla

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FLOW CYTOGENETICS: PROGRESS TOWARD CHROMOSOMAL ABERRATION DETECTION

A.V. Carrano, J.W. Gray and M.A. Van Dilla

Biomedical Division, Lawrence Livermore Laboratory,

Livermore, California U.S.A.

All inquiries concerning this manuscript should be addressed to:

Dr. A.V. Carrano

Biomedical Division L-452

Lawrence Livermore Laboratory

Livermore, California 94550 U.S.A.

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Introduction

Over the past several years it has become increasingly evident that mankind is being exposed to a wide variety of clastogenic agents, i.e., physical or chemical agents which are capable of breaking chromosomes. This realization has placed a considerable strain on the scientists responsible for determining the genetic consequences of such exposure largely because conventional methods of chromosomal aberration analysis are ill-suited to large scale population studies. They are subjective, slow and tedious. Semi-automated and automated systems have been proposed as means of assisting or supporting the cytogeneticist for aberration scoring (Castleman et al., 1976; for a comprehensive review see Mendelsohn, 1976) but in general, they lack the high throughput or accuracy necessary for population monitoring.

For the past three years we have been developing the instrumentation and biological methodology for the analysis of isolated metaphase chromosomes by flow cytometry (Gray et al., 1975a; Gray et al., 1975b; Carrano et al., 1976; Carrano, Van Dilla and Gray, 1977). This technique permits the examination of approximately 1000 individual metaphase chromosomes per second. The parameter quantified is the amount of fluorescence emitted by each stained chromosome as it crosses a beam of intense laser light. The data output is a distribution of chromosome frequency versus fluorescence intensity which, for ethidium bromide stained chromosomes constitutes a DNA flow karyotype. This approach offers the advantages of objectivity and speed; to establish a single karotype the time required from initiation of data collection through data analysis is approximately 20 minutes. We describe below the results of our continuing efforts to apply this technology to the

analysis of chromosomal aberrations in a model system, the Chinese hamster cell line M3-1. Consideration is given to both homogeneous aberrations, i.e., events which are present and identical in every cell (e.g., heritable translocations) and heterogeneous aberrations, i.e., events which are usually not heritable and occur at random in a cell population (e.g., dicentrics and deletions).

Materials and Methods

Cell culture and chromosome isolation. The Chinese hamster M3-1 cells are cultured in 650 cm^2 surface area glass roller bottles in 150 ml of Minimimal Essential Medium supplemented with NCTC-135 (final concentration, 10%), fetal calf serum (final concentration, 15%), and gentamycin and chlorotetracycline as antibiotics. Under these conditions the generation time is 13 hours. For chromosome isolation, cells are seeded in the same roller bottles at a density of $5 \times 10^4/cm^2$ and grown for approximately 44 hours. At this time the medium is removed and fresh medium containing colcemid (0.037 µg/ml, final concentration) is added for a period of 5 hours. Following mitotic cell accumulation the roller bottles are rotated at 300 rpm for for six minutes on a culture rotator (*Talandic Corporation*, Pasadena, California U.S.A.). The medium is then decanted, the detached cells are counted and aliquots of 5×10^6 cells are pelleted in a centrifuge. Approximately 2×10^7 cells (mitotic index >90%) are harvested from each roller bottle.

The chromosome isolation procedure has been adopted from Wray and Stubblefield (1970). The mitotic cell aliquots are resuspended in potassium chloride (0.075 M) at 4°C for 30 minutes, pelleted, and

resuspended in 0.5 ml of pH 6.7 chromosome isolation buffer consisting of 0.1 mM piperazine-N, N'-bis 2-ethanesulfonic acid, 1 M hexylene glycol and 1 mM calcium chloride. The chromosome isolation buffer was modified during the course of these experiments and now consists of 25 mM Trizma base (adjusted to pH 7.5 with hydrochloric acid), 0.75 M hexylene glycol, 0.5 mM calcium chloride and 1.0 mM magnesium chloride. The 0.5 ml suspension of mitotic cells is sheared at 4°C in a Virtis homogenizer to disrupt the cell membrane and free the metaphase chromosomes. Staining is accomplished by adding to the sheared suspension an equal volume of 0.2 mM ethidium bromide (EtBr) in the same isolation buffer. The final suspension contains approximately 10^6 metaphase chromosomes in 1 ml of buffer at a final EtBr stain concentration of 0.1 mM.

Flow cytometry and sorting. The isolated and stained chromosomes are analyzed on the Lawrence Livermore Laboratory flow cytometer (FCM) or sorted on the Becton-Dickinson (B-D) flow sorter. For measurement of the relative fluorescence intensity on the FCM, about 5×10^5 chromosomes are analyzed at a rate of approximately 1000/sec. The fluorescence distributions are computer fitted by a least squares method using multiple Gaussian functions superimposed on an exponential function. Each Gaussian function represents a distinct group of chromosomes; the Gaussian mean representing the mean chromosome group fluorescence and the area representing the chromosome group frequency of occurrence. The exponential function is a measure of the background, i.e., fraction of the total area in the distribution underlying the chromosomal peaks (Moore II, 1975; Carrano, Van Dilla and Gray, 1977). We ascribe this background to fluorescent debris and/or chromosomal

fragments. The output of this fitting program includes the mean, area, and coefficient of variation of each chromosome peak, and the background area.

In order to preserve the chromosome morphology during flow sorting, the chromosome isolation buffer is used as the sorting fluid. Approximately 10,000 chromosomes per sort are collected directly on microscope slides and fixed by slowly dropping 3:1 (absolute methanol: glacial acetic acid) onto the chromosomes, followed by two further changes of the same fixative. The slides are then stained with a 7.5% Giemsa solution in distilled water.

Results

The Flow Karyotype

The karyotype of clone 650A of the Chinese hamster M3-1 cell line consists of 23 chromosomes. Chromosomal banding analysis distinguishes 14 distinct types (Gray et al., 1975a). These are indicated in Table 1. If each of the chromosome types possessed a unique amount of DNA then one would expect 14 distinct peaks in the FCM generated chromosomal DNA distribution. Fig. 1 shows the distribution actually obtained after staining the isolated chromosomes with ethidium bromide. Nine distinct peaks are present indicating that some chromosomes have very similar amounts of DNA which are not resolved in this distribution. The chromosome types that constitute each peak have been determined by flow sorting and subsequent banding analysis as well as by independent scanning cytophotometric measurements of chromosomal DNA content (Gray et al., 1975a and Carrano, Van Dilla and Gray, 1977). The chromosome types ascribed to the peaks in the fluorescence distribution along with

computer estimates of peak means (approximately proportional to DNA content) and peak areas (proportional to relative frequency of occurrence) are shown in Table 1. There is excellent agreement between the actual chromosome group frequencies determined by banding analysis of metaphase cells and that determined from the FCM distribution. The relative DNA content and chromosomal frequency therefore constitute the quantitative DNA flow karyotype.

Analysis of Homogeneous Aberrations

If both the relative DNA content and chromosomal frequency can be established for each peak in the FCM distribution, it should be possible to karyotype a cytogenetically identical cell population. To test this hypothesis, we acutely irradiated clone 650A cells with either 300 or 1000 rad of 300 kVp X rays and immediately cloned several subpopulations. These clones were allowed to grow for approximately two months in culture at which time their chromosomes were isolated for FCM analysis. The flow karyotypes of two clones, 650AAA and 650AB, derived by this protocol, are shown in Figs. 2A and 2B, respectively. The results of the computer fit to these distributions are given in Table 2.

From this figure and the Table it is evident that, for both clones, peaks D through I are unaltered either in relative mode or area compared to clone 650A. The FCM distribution of clone 650AAA chromosomes (Fig. 2A) differs from that of clone 650A (Fig. 1) in that two new peaks, T_1 and T_2, are present and areas of peaks B and C are changed. Peaks B and C have each lost one chromosome while peaks T_1 and T_2 each contain one chromosome. If the amount of DNA in chromosome 1 is expressed on an arbitrary scale as 100 units, the relative means show that the chromosome in peak T_1 has 8 units less DNA than the chromosome in peak B

while the chromosome in peak T_2 has 10 units more than the chromosomes in peak C. Thus there appears to have been reciprocal exchange involving chromosome 2 in peak B and chromosome 4 or t(X;5) in peak C. The net effect is such that the chromosome from peak B lost 8–10 units of DNA and the chromosome from peak C gained a like amount of DNA. The new peaks T_1 and T_2 contain the derived chromosomes and peaks B and C, from which the derived chromosomes originated, are reduced in area. This prediction from the flow karyotype, of a reciprocal translocation between chromosomes in peaks B and C is confirmed by the Giemsa-banded karyotype of metaphase chromosomes (Table 2) that demonstrates a reciprocal translocation between chromosomes 2 and 4.

The flow karyotype of clone 650AB (Fig. 2B) has been previously reported (Gray *et al.*, 1975b) and is summarized here. In this case compared to clone 650A, (Fig. 1) peaks A and C have each lost one chromosome, peak B has gained one chromosome and peak T_3 has been formed with one chromosome. On a scale of 100 units for chromosome 1, the chromosome in peak T_3 has 12 units more DNA than the chromosomes of peak C. The additional chromosome in peak B has 15 units less DNA than the chromosome in peak A. These results again suggest a reciprocal translocation of about 12 to 15 units of DNA involving chromosome 1 in peak A and either chromosome 4 or t(X;5) in peak C. Banding analysis confirms the translocation to be reciprocal between chromosomes 1 and 4 and the derivative chromosomes have been identified in peaks B and T_3.

Analysis of Heterogeneous Aberrations

Aberrations induced by clastogenic agents include asymmetrical and symmetrical exchanges, deletions, and inversions of chromosomal material. They normally occur at random so that a population of exposed

cells contains many cells with different abnormal karyotypes in addition to the unaffected cells with a normal karyotype. Unless selection had intervened, one would not expect the flow karyotype of these cells to exhibit clearly defined peaks other than those exhibited by the prevalent normal karyotype. In order to empirically determine the shape of the FCM distribution of chromosomes isolated from cells with heterogeneous aberrations, we acutely irradiated clone 650A with 350 kV X rays. Chromosomes were isolated from these cells within 7 hours after irradiation, a period of time in which only cells from G_2 and S would be arriving at metaphase. Hence the isolated chromosomes would contain chromatid aberrations.

The FCM distributions of clone 650A chromosomes exposed to 75 rad and 150 rad of X rays are shown in Figs. 3A and 3B, respectively. Comparing these distributions to the unirradiated clone 650A distribution in Fig. 1, it is evident that the background, that is, the area underlying the peaks, is increased after irradiation and that this increase is dose dependent. This result is expected for random chromosome breakage.

If breakage is random and all breakpoints have an equal probability of exchange, then the larger chromosomes would show more exchanges. Thus, exchanges should predominate in large chromosomes (at high DNA amounts) and fragments at small DNA amounts. To test this, cells from clone 650A were given an acute dose of 300 rads of X rays and chromosomes were isolated from metaphase cells accumulated during a 5 h colcemid arrest from 15 to 20 hours after irradiation. This metaphase population consists of cells that were in G_1 at the time of irradiation or were in G_2-M and are now in their second mitosis. Thus the

aberrations are of the chromosome type. The FCM distribution generated by these ethidium bromide stained chromosomes is shown in Figs. 4 and 5. Chromosomes from each peak and valley of the distribution were sorted onto microscope slides, stained and scored visually for aberrations. The aberration frequency is expressed as dicentrics or deletions per scored chromosome. Two thousand chromosomes were scored for each sort.

The frequency of dicentric chromosomes as a function of DNA content is shown in Fig. 4. These aberrations predominate at high DNA amounts and their frequency is increased in the valleys compared to the peaks. This is simply a reflection of the fact that adjacent peak and valley regions have similar numbers of dicentrics but very different numbers of normal chromosomes, and the frequency is defined as the ratio of dicentrics to total chromosomes. Fig. 5 shows the frequency of chromosome deletions (acentric fragments) as a function of DNA content. These aberrations predominate at low DNA amounts and their frequency is increased in the valleys compared to the peaks for the same reasons as for dicentrics. These results from flow sorting strengthen the hypothesis that the increased background in the flow karyotype following irradiation is due to the presence of chromosomal aberrations. Other supporting evidence has been discussed previously (Van Dilla, Carrano and Gray, 1976; Carrano, Van Dilla and Gray, 1977).

These results suggest a quantitative relation between the background of the FCM distribution and the frequency of aberrations in a cell population. Figure 6 summarizes the results of seven independent experiments to determine whether a quantitative correlation exists. For these experiments clone 650A cells were irradiated with doses of X ray from 25 to 300 rad. Prior to chromosome isolation, a portion of the

metaphase cells were prepared on microscope slides for aberration analysis. Chromosomes were isolated from the remaining cells, stained with ethidium bromide and analyzed on the FCM. The background from each distribution was obtained from the computer fit. The mean squared residual, also obtained from the computer analysis, is the sum of the squared differences between the data points and the computer generated fit divided by the number of channels over which the data was collected; it is an indication of the goodness of fit between the data points and the mathematical functions used to fit these data points.

Figure 6A demonstrates an increase in the background as a function of the aberration frequency. A 50% increase in the background occurs at an aberration frequency of about 0.8 per cell. We also observed that the mean squared residual (Fig. 6B) decreases with increasing aberration frequency for unexplained reasons. Unfortunately the experiment to experiment coefficient of variation for the background is approximately 13% and for the mean square residual is about 6%. Thus the quantitative estimation of the metaphase cell aberration frequency from the increased background is not yet possible. At present these parameters serve as a qualitative indication of clastogen damage for high levels of exposure; it is not likely that the sensitivity of this method will be increased to the extent necessary for monitoring populations exposed to low levels of clastogens. For this purpose, we have suggested (Van Dilla, Carrano and Gray, 1976) a slit-scan approach to chromosomal flow cytometry (see Discussion).

Discussion

Flow analysis of isolated chromosomes has been demonstrated in at least three laboratories (Gray et al., 1975a; Stubblefield, Cram, and Deaven, 1975; Deaven, Stubblefield and Jett, 1976; Otto and Oldiges, 1977). The preparative technique is rapid. From mitotic cell collection to stained isolated chromosomes requires 1.5 h. The flow karyotype can be generated by the FCM in about 10 min and computer analysis of the distribution requires a further 10 min. It is possible therefore to establish a karyotype in approximately 2 h, considerably faster than the traditional "cut and paste" method. Thus flow analysis offers the potential for rapidly screening a large number of samples. It would be of extreme utility in screening animal or human populations for heritable aberrations resulting from exposure to environmental pollutants. In the investigations reported here we show that DNA flow karyotyping can be applied to the analysis of homogeneous aberrations. In an earlier study it was found that flow chromosome analysis could be applied to detect mosaicism (Van Dilla, Carrano and Gray, 1976; Carrano, Van Dilla and Gray, 1977). It was possible in that study, to detect the presence of the translocated chromosome, der(4), in clone 650AB when it was present in a mixed population consisting of 5% clone 650AB cells and 95% clone 650A cells.

In principle, flow karyotype analysis should detect any homogeneous chromosomal alterations that involve a gain, loss or exchange of unequal amounts of DNA. Reciprocal exchanges of equal DNA amounts and inversions are not detectable. In addition, the detection of chromosomal exchanges of unequal DNA amounts, deletions or additions is very much dependent upon the resolution of the chromosomes on the FCM.

In clones 650AAA and 650AB, for example, we were unable to state definitively from the flow karyotype whether chromosome 4 or the t(X;5) chromosome was involved in the translocation since the two were found in the same peak. In recent experiments, using the dye 33258 Hoechst, we have been able to resolve these chromosomes as well as the chromosomes with similar DNA contents in peaks E-F, H, and I of clone 650A. These results will greatly facilitate flow karyotype analysis with this species.

There are at least three approaches to the analysis of heterogeneous chromosomal aberrations. The first depends solely on a quantitative estimate of a dose dependent alteration in one of the parameters derived from the FCM flow karyotype, e.g., the background or mean square residual. Unfortunately this approach has up to the present, served only as a qualitative indicator of chromosome damage. Further progress must be made on the preparative, cytochemical and instrumental procedures for handling isolated chromosomes to minimize the sample-to-sample and experiment-to-experiment variation in chromosome shear, in the cytochemistry and in the optimization of the fluorescence signal-to-noise ratio.

The second approach to heterogeneous aberration detection is based upon flow sorting enriched fractions of aberrant chromosomes onto microscope slides followed by conventional cytogenetic analysis. The feasibility of this method is dependent upon the degree of enrichment of the aberrant chromosomes. In order to relate the aberration frequency in the sorted chromosomes to that in metaphase cells precise calibration is necessary. For example, one may desire to know the quantitative relation between the frequency of dicentrics in the valleys to the

metaphase cell frequency. If such a correlation existed it would be possible to score the aberrant chromosomes sorted from a single valley to attain the metaphase cell frequency. The scoring of the sorted chromosomes could either be performed manually by a trained cytogeneticist or might even be facilitated by slide-based scanning instrumentation. The isolated chromosomes eliminate one of the major obstacles to the image scanning systems; namely, the discrimination between overlapping chromosomes or chromatids and a true dicentric chromosome in a single metaphase cell. The isolated chromosome morphology is preserved during sorting and the chromosome density on the slide can be adjusted to eliminate overlap. We believe that, if a significant enrichment of aberrant chromosomes can be obtained by flow sorting, this method holds promise for a semi-automated or fully automated system for clastogen screening.

The third approach to the detection of aberrations focusses on the dicentric chromosome and requires further instrument development. The general principle calls for a flow cytometer capable of detecting the centromere(s) in isolated chromosomes. In the proposed instrument each stained chromosome would pass lengthwise through a very thin laser beam. The resulting fluorescence signal would be a chromosome profile with a dip at the centromere region(s). The number of dips per chromosome profile would be determined; two such dips per chromosome would indicate a dicentric. Alternatively, fluorescent stains specific for the centromere regions might also be developed. Such instrumentation is currently being designed in our laboratory.

Finally, purified chromosomes are important for biochemical analysis. Studies conducted with clone 650A of the Chinese hamster

chromosomes show that DNA can be recovered from isolated sorted chromosomes, that such DNA can be transcribed and that the transcribed messenger can be hybridized to chromosomes in situ (Gavin, Scherberg and Carrano, 1977). Such studies are potentially applicable to gene mapping. For example, unique sequence messenger RNA could be initially hybridized (in solution) to DNA from highly purified sorted chromosome fractions to map the gene to a specific chromosome. Once the chromosome is identified it is then feasible to obtain translocation or deletion variants of that chromosome which could be uniquely distinguished and sorted by the flow systems. This would further localize the gene to a specific chromosome region.

Flow chromosome analysis has potential both for karyotype analysis and biochemistry. We hope that this manuscript stimulates sufficient interest in other laboratories so that we might share the excitement of future development.

Summary

Using clonal derivatives of the Chinese hamster M3-1 cell line, we demonstrate the potential of flow systems to karyotype homogeneous aberrations (aberrations which are identical and present in every cell) and to detect heterogeneous aberrations (aberrations which occur randomly in a population and are not identical in every cell). Flow cytometry (FCM) of ethidium bromide stained isolated chromosomes from clone 650A of the M3-1 cells distinguishes nine chromosome types from the fourteen present in the actual karyotype. X-irradiation of this parent 650A clone produced two sub-clones with an altered flow karyotype, that is, their FCM distributions were characterized by the addition of new peaks and alterations in area under existing peaks.

From the relative DNA content and area for each peak, as determined by computer analysis, we predicted that each clone had undergone a reciprocal translocation involving chromosomes from two peaks. This prediction was confirmed by Giemsa-banding the metaphase cells. Heterogeneous aberrations are reflected in the flow karyotype as an increase in background, that is, an increase in area underlying the chromosome peaks. This increase is dose dependent but, as yet, the sample variability has been too large for quantitative analysis. Flow sorting of the valleys between chromosome peaks produces enriched fractions of aberrant chromosomes for visual analysis. These approaches are potentially applicable to the analysis of chromosomal aberrations induced by environmental contaminants.

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Figure Legends

Figure 1. The flow karyotype of Chinese hamster M3-1 clone 650A. The isolated chromosomes were stained with ethidium bromide. Approximately 5×10^5 chromosomes were analyzed to obtain this distribution. The fit line generated by computer analysis is drawn through the data points.

Figure 2. The flow karyotype of Chinese hamster M3-1 clone 650AAA (Fig. 2A) and clone 650AB (Fig. 2B). The chromosomes were stained with ethidium bromide. The two distributions were analyzed at different amplifier gain settings and thus the length of the abscissas differ. The computer generated fit line is drawn through the data points.

Figure 3. The flow karyotype of X-irradiated Chinese hamster M3-1 clone 650A chromosomes. A. Clone 650A cells received 75 rad of X rays seven hours prior to chromosome isolation. B. Clone 650A cells received 150 rad of X rays seven hours prior to chromosome isolation. The computer generated fit line is drawn through the data points.

Figure 4. The frequency of dicentric chromosomes in each peak and valley of the flow karyotype of irradiated (300 rad) clone 650A cells. Chromosomes were isolated 20 h after irradiation.

Figure 5. The frequency of chromosome deletions in each peak and valley of the flow karyotype of irradiated (300 rad) clone 650A cells. Chromosomes were isolated 20 h after irradiation.

Figure 6. A. The relation between the background of the flow karyotype and the frequency of aberrations in the metaphase cells from which the chromosomes were isolated. B. The relation between the mean square residual and the aberration frequency. Each data point is pooled from seven independent experiments. A least squares linear regression line is drawn through each data set.

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Table 1. Comparison between the Giemsa-banded and DNA flow karyotype of the Chinese hamster M3-1 clone 650A

Giemsa-banded karyotype		DNA flow karyotype		
Chromosome type	Chromosome frequency	Peak designation	Relative mode	Chromosome frequency
1	2	A	1.0	1.92
2	2	B	0.86	1.79
4, t(X;5)	3	C	0.54	3.03
5	1	D	0.46	1.02
6,7,Y	5	E,F[a]	0.35	5.29
8	2	G	0.29	2.02
9,M1	3	H	0.23	3.02
10,11,M2	5	I	0.15	4.90

[a] These peaks are considered together since it is often difficult to resolve their individual modes and areas.

Table 2. The DNA flow karyotypes of Chinese hamster clones 650 AA and 650AB

Clone 650AAA				Clone 650AB			
Peak designation[a]	Chromosome type[b]	Relative mode	Chromosome frequency	Peak designation[a]	Chromosome type[b]	Relative mode	Chromosome frequency
A	1	1.0	2.12	A	1	1.0	0.93
B	2	0.86	0.93	B	2,der(1)	0.85	2.80
T_1	der(2)[c]	0.78	1.11	T_3	der(4)	0.65	1.19
T_2	der(4)	0.63	1.14	C	4,t(X;5)	0.53	2.30
C	4,t(X;5)	0.53	2.02	D	5	0.46	0.97
D	5	0.46	0.88	E,F	6,7,Y	0.36	4.81
E,F	6,7,Y	0.36	5.03	G	8	0.29	2.17
G	8	0.29	2.29	H	9,M1	0.23	3.16
H	9,M1	0.23	2.89	I	10,11,M2	0.15	4.67
I	10,11,M2	0.15	4.59				

[a]The peak designation, relative mode and chromosome frequency were determined from analysis of the FCM distributions in Fig. 2.

[b]The chromosome type was determined from Giemsa-banded metaphase cells.

[c]The term der is used to indicate a derivative chromosome, in this case, a structurally rearranged chromosome resulting from a translocation. The number in parenthesis indicates the chromosome from which the centromere was derived (Paris Conference, 1971).

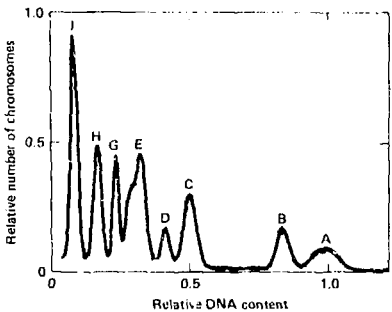

Fig. 1

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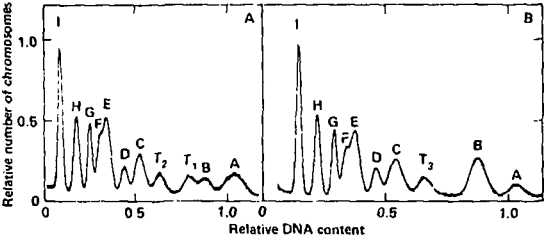

Fig. 2

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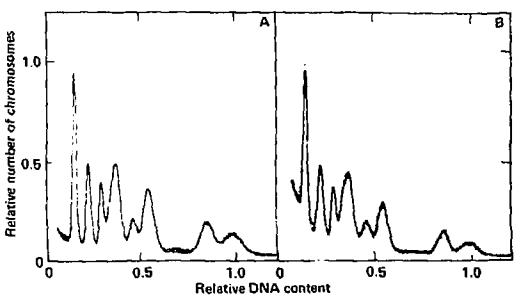

Fig. 3

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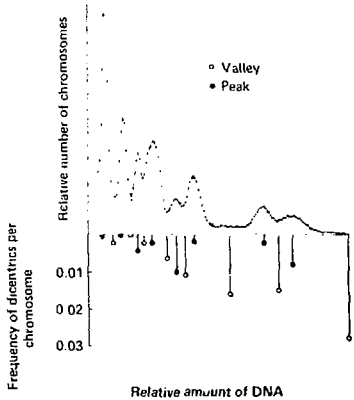

Fig. 4

Carrano, Gray, and Van Dilla

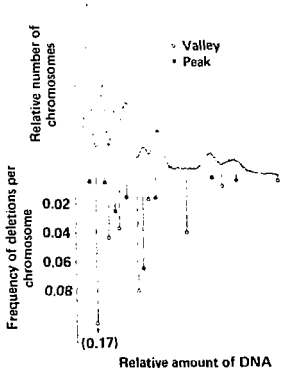

Fig. 5

Carrano, Gray, and Van Dilla

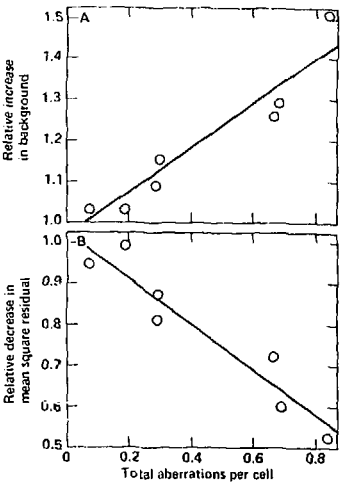

Fig. 6

Carrano, Gray, and Van Dilla