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SUBAL C. CHATTOPADHYAY

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CHROMOSOME ABERRATIONS INDUCED BY 7,12-DIMETHYLBENZ(A)ANTHRACENE IN RAT BONE MARROW CELLS - THEIR RELATIONSHIP TO CHROMOSOME BANDS AND THE INFLUENCE OF ERYTHROPOIETIN -*

SUBAL C. CHATTOPADHYAY

Second Division, Department of Pathology
Kobe University School of Medicine

INDEXING WORDS

chromosome aberration; 7,12-dimethylbenz(a)anthracene; carcinogen; chromosome breakage; erythropoietin; hormone

SYNOPSIS

The distribution of chromosomal aberrations (CA) along all chromosomes was studied in bone marrow cells of rats treated with 7,12-dimethylbenz(a)anthracene (DMBA) using the Q-banding technique. With respect to chromosomal localization, the largest telocentric chromosome (#2) was most susceptible to DMBA. The distribution of CA on chromosomal bands was non-random and the bands 1q22, 2q16, 2q22, 2q24, 3q24, 4q22, 4q34, 5q12, 6q24, 11q12, and 14q11 were involved in CA at a statistically significant

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Abbreviations used: DMBA=7,12-dimethylbenz(a)anthracene; TMBA=7,8,12-trimethylbenz(a)anthracene; CA=Chromosome aberration(s).

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Author's name in Japanese : スバル・チャンドラ・チャトパタイヤ

level. Most of them were Q-negative bands and some Q-positive bands and interfaces were also involved. Comparison with the results obtained by the direct measurement method suggested that mapping along banded chromosomes caused false results probably because many CA occurring in Q-bands or interbands may be read as occurring in Q-negative bands. An important role of growth stimuli such as erythropoietic stimuli in non-random distribution of CA in certain chromosomal bands in bone marrow cells was also demonstrated.

INTRODUCTION

Although considerable work has been done on the interaction between chemical carcinogens and cellular DNA, little is known about the specific interaction during carcinogenesis.[24, 34] One possible way to elucidate the specific interaction during carcinogenesis is to understand the action of chemical carcinogens at the chromosomal level.

Sugiyama showed in 1971 that hydrocarbon carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA) and 7,8,12-trimethylbenz(a)anthracene (TMBA) acted non-randomly on chromosomes[28, 33] and induced rat leukemias with non-random chromosomal changes involving the largest telocentric chromosome (#2).[26, 27, 31] The same chromosomal changes have also been reported in DMBA-induced sarcomas[12] and carcinomas.[1] Moreover, some 1-butyl-1-nitrosourea(BNU)-induced leukemias also have the same chromosomal changes.[34]

Additional studies[32] have shown that several structurally different chemical carcinogens and mutagens such as DMBA, TMBA, urethane, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF2), and Mitomycin C induced CA and SCE which were similarly distributed along chromosomes suggesting a common mode of action of these chemicals on chromosomes; the CA induced with these chemicals along #1 and #2 chromosomes were distributed non-randomly and the peak distribution of DMBA-induced CA at 6 hr roughly corresponded to the Q-positive bands 1q31, 2q21 and 2q25 by direct plotting of the relative site of chromosome breaks along non-banded chromosomes.[32] However, many reports from other laboratories indicate that radiation- and chemically-induced CA are non-randomly

distributed mainly in G- or Q-negative bands or in interfaces between a chromosome band and the neighboring interband.

Therefore, we planned to map the susceptible sites for DMBA along chromosome complements of the rat using a banding technique because more precise localization was expected with this technique. Harvest 6 hr after DMBA treatment was again used in the studies because the maximum non-random distribution was demonstrated at this time-period[28, 33] and the results could easily be compared with previous results.

On the other hand, from association of CA with the late DNA replicating regions of chromosomes,[32] the DNA replication pattern was considered important for the susceptibility of these regions. The role of other factors such as growth stimuli (erythropoietin, EP) in distribution of CA was also studied.

MATERIALS AND METHODS

Animals

Non-inbred male Long-Evans rats, 30-day-old, originally separated from the colony in Ben May Laboratory for Cancer Research (Prof. Charles Huggins), the University of Chicago, were used. They were given a commercial ration, CMF (Oriental Yeast Co., Tokyo) and water *ad libitum*.

Chemical carcinogens

DMBA (Eastman Organic Chemicals, Rochester, N.Y.) was purified by silica gel column and recrystalization and DMBA emulsion was prepared in our laboratory with a Manton-Gaulin submicron disperser. It contained DMBA at a concentration of 5mg/ml. The emulsion was given intravenously into the tail vein of rats at a dose of DMBA (50mg/kg body weight) 6 hr before sacrifice.

Chromosome specimens

Chromosome specimens were prepared directly from the bone marrow. The animals were given 0.3mg of colchicine intraperitoneally 1 hr before sacrifice. They were killed by aortic puncture and the bone marrow was removed from the femur. The bone marrow cells suspended in physiological saline were treated with 0.075

mole KCl at 37C for 15 min, fixed in Carnoy's solution (methanol: acetic acid=3:1) 3 times, and spread on glass slides by the air-drying method. The specimens were stained with 2% Giemsa in 0.01M phosphate buffer (pH6.8) for 15 min. Chromosomes and chromosome bands were classified according to the nomenclature for chromosomes and G-bands of Norway rats proposed by Levan.[13)]

Chromosome aberrations and mapping

Chromosome aberrations (CA) consisted of chromosome and chromatid breaks with chromatid discontinuity and gaps with a clear achromatic lesion and no evidence of discontinuity. This definition of breaks and gaps in our system nearly fulfils that of Wolff and Bodycott[35)] proposed for defining chromosome and chromatid breaks.

For intrachromosomal distribution of CA along the long arms of chromosomes #1 and #2 and other chromosomes, two methods were used; a) the relative position expressed as a percentage of the proximal and distal ends of each CA from the centromere was plotted along the chromosome axis as described first by Sugiyama[28)] and b) the DMBA-induced CA were also plotted on Giemsa(G)- or quinacrine(Q)-banded chromosomes for comparison with the results obtained by the above measuring method. The Seabright trypsin method[23)] was used for demonstration of G-bands. A vertical illumination fluorescence microscope (Fluorophoto, Nikon) was used for observation of Q-bands.

Erythropoietic stimuli

Hemopoiesis was stimulated by blood letting and suppressed by polycythemia induction as described by Sugiyama.[29)] These treatments were given 24 hr before DMBA injection to ensure stable physical condition. Anemia was induced by drawing 2.5ml of whole blood/100g body weight by cardiac puncture and polycythemia by intravenous injection of 3ml of washed red blood cells per 100g of body weight. The red blood cells were obtained from adult males of the same strain, washed 3 times by centrifugation in physiologic saline to remove the buffy coat layer and blood plasma, and finally suspended in physiologic saline at a concentration of 79 ± 2% in packed cell volume. Bone marrow cells were stimulated by intraperitoneal injection of 6U of step 3 sheep EP

(Connaught Medical Laboratories, Ontario, Canada) and then dissolved in physiologic saline at a concentration of 10U/ml. The hematocrit values of the aortic blood of each animal at the time the animal was killed were determined by the microhematocrit method after the blood was spun at 12,000rpm for 5 min with a microhematocrit centrifuge.

DNA replication pattern

Normal bone marrow cells (10^6cells/ml) were incubated for 11.5 hr in 5-bromodeoxyuridine(BrdU, 2μg/ml)-containing F12 medium (Nissui Seiyaku, Tokyo) and thereafter transferred into thymidine(dT)-containing F12 medium (Nissui Seiyaku, Tokyo) incubated for 1.5 hr, and collected for chromosome specimens. The cells were treated with 2μg colchicine/ml for 1 hr before harvest. Pulse-labeling with dT at other periods of the cell cycle was also performed. The dT-labeled regions were demonstrated by the modified Hoechst 33258-Giemsa method.[25] In this modification, blacklight irradiation of chromosome specimens was followed by incubation in 6M urea for 15-30 min at 50C, and then the specimens were stained in Giemsa solution.

RESULTS

Plotting of CA by the measuring method

DMBA-induced CA at 6 hr were plotted along the non-banded #1 and #2 chromosomes (Fig. 1a). One moderate peak was found in 1q31 region of #1 chromosome and two prominent peaks in 2q21 and 2q25 of the #2 chromosome. These peaks were already reported to be time-dependent or stage-specific within a cell cycle and reproducible.[28]

Plotting along G-banded #1 and #2 chromosomes

In total 100 DMBA-induced CA at 6 hr were plotted along the G-banded chromosomes. CA in 1q31 were plotted in 1q22/31/32 and the peak in 2q21 tended to split into 2 further peaks in 2q16 and 2q22 and those in 2q25 into 2q24 and 2q26 respectively (Fig. 2). Another minor peak was found in 2q34.

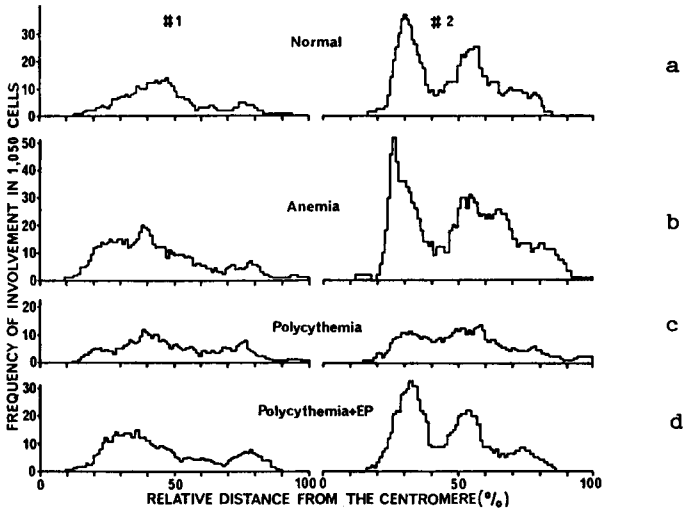

Fig. 1 Frequency distribution of DMBA-induced CA along the long arms of chromosomes #1 and 2 under various hemopoietic stimuli. Although each curve was made by plotting 100 CA along each chromosome, the curves were redrawn to show the relative frequency in a total of 1,050 cells from 6 animals in each group. The 0 and 100% represent the centromere and the terlomere of these chromosomes, respectively. Note prominent amplification of distribution curves in chromosome #2 under hemopoietic stimuli (b and c) compared with that in suppressed hemopoiesis (c). *Abscissa*: % distance from the centromere; *Ordinate*: frequency of CA in each chromosome segment expressed by absolute number of CA found in 1,050 cells.

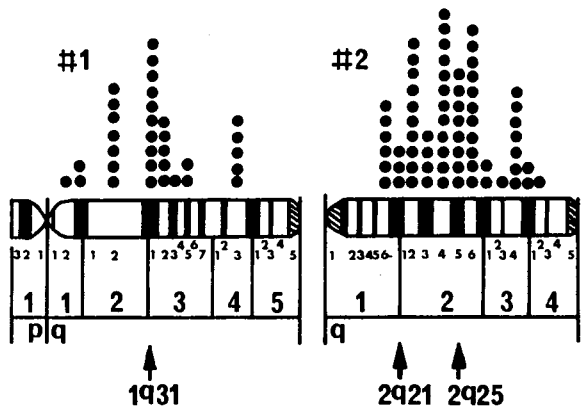

Fig. 2 Frequency distribution of 100 CA induced 6 hr after DMBA treatment along G-banded chromosomes #1 and #2.

Plotting along Q-banded chromosome complements

Analysis of distribution of DMBA-induced CA along Q-banded chromosome complements was carried out 6 hr after DMBA treatment. Well exhibited Q-banding metaphases were selected for the present analysis and where the quality of Q-bands was not reproducible, plotting of the breakage points was not considered. A total of 427 CA were mapped to individual rat chromosomes (Fig. 3).

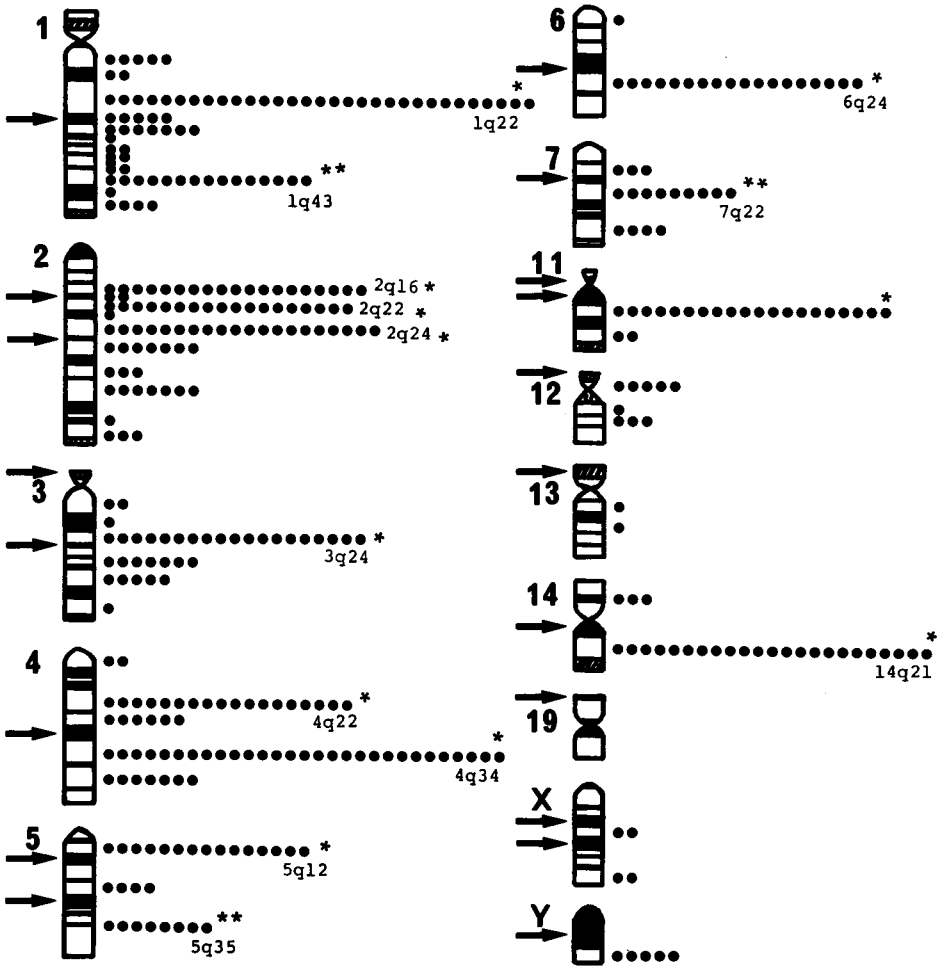

Fig. 3 Frequency distribution of a total of 427 CA on G-banded chromosomes of bone marrow cells of the rat examined 6 hr after DMBA treatment (50mg/kg body weight). *Significant only at the *per*-band bases. Late DNA-replicating regions are shown by arrows. **Significantly susceptible sites ($p<0.05$).

Statistical analyses for non-random distribution were assessed by χ^2-test for the incidence of CA per band for a total of 557 bands and for the incidence of CA per region for a total of 72 regions arbitrarily divided. The latter was essentially based on the length of each chromosomal band as schematized by Levan.[13)]

Table 1 indicates the incidence of CA on each chromosome complement. The non-randomness in frequency of each chromosome

Table 1 Incidence of CA in each chromosome complement.

Chromosome number	Relative length	Actual No. of CA	Expected No. of CA	Relative incidence	Statistics (χ^2-value)	Significant difference*
#1	51	78	39	1.53	15.06	+ $p<0.005$
#2	49	81	38	1.65	18.05	+ $p<0.005$
#3	37	35	28	0.94	0.83	ns
#4	37	62	28	1.67	14.35	+ $p<0.005$
#5	32	27	25	0.84	0.08	ns
#6	28	19	21	0.68	0.10	ns
#7	26	16	20	0.62	0.46	ns
#8	25	9	19	0.36	3.69	ns
#9	25	12	19	0.48	1.63	ns
#10	25	7	19	0.28	5.71	- $0.01<p<0.05$
#11	20	22	15	1.10	1.38	ns
#12	18	9	14	0.50	1.11	ns
#13	23	2	18	0.08	13.10	- $p<0.005$
#14	19	26	15	1.37	3.10	ns
#15	22	8	17	0.36	3.33	ns
#16	19	4	15	0.21	6.51	- $0.01<p<0.05$
#17	19	0	15	0.00	15.26	- $p<0.005$
#18	16	1	12	0.06	9.45	- $p<0.005$
#19	14	0	11	0.00	11.14	- $p<0.005$
#20	11	0	8	0.00	8.07	- $p<0.005$
X	26	4	20	0.15	10.97	- $p<0.005$
Y	15	5	11	0.33	2.29	ns
Total	557	427	427	(f=21)	137.46	$p<0.005$

*ns: Not significant.

was statistically significant as a whole ($p<0.005$) and CA were increased in #1, #2, and #4 chromosomes and reduced in #9, #13, #16-#20, and X at a significant level ($p<0.01$).

The following data clearly revealed that breakage points were mainly distributed in Q-negative bands and that the juxtaposition of Q-positive and Q-negative bands (interfaces) also manifested the clustering of CA. Among 427 CA, 78 were located in #1 chromosome. The band 1q22 in the long arm of #1 was significantly susceptible ($p<0.005$); 31 (39.74%) of 78 CA in #1 were located in this band. Another Q-negative band 1q43 also accumulated CA significantly ($p<0.005$) when evaluated by the *per*-band basis. Analysis of 81 site-specific CA on chromosome #2 indicated that there were 3 susceptible Q-negative bands identified as 2q16, 2q22, and 2q24. The incidence in each band was 19, 18, and 20 respectively. The distribution frequency of potential breaks along the long arm of chromosome #3 indicated that only the band 3q24 was significantly susceptible ($p<0.005$). The site specific CA analysed on chromosome #4 showed that 18 and 29 out of 62 CA were assigned to 4q22 and 4q34 respectively. On #5, 15 and 8 CA were located in 5q12 and the interface 5q35/36, respectively. Out of 19 CA assigned to chromosome #6, 18 were located in band 6q24. Out of 16 CA in #7, 9 were clearly discernible in band 7q22. The evaluation revealed a significant difference only in the *per*-band basis ($0.01<p<0.05$). On the other hand, in #8, the band 8q22 was mainly involved in #8 in a high incidence of CA although both evaluations revealed that #8 involvement was not significant. Chromosomes #9 and #10 had no specifically vulnerable sites based on statistical analysis. The band 11q12 was mainly involved in CA in chromosome #11. Twenty out of 22 CA were preferentially localized in the band 11q12. A few CA were not only localized to the long arm of chromosome #12 such as bands 12q12 and 12q14 but also extended to the short arm as evidenced by the presence of CA in bands such as 12p11. Chromosome #13 was not highly susceptible. Chromosome #14 was highly susceptible to the clastogenic action of DMBA. Both short and long arms were involved with CA. A careful examination of break points revealed that 20 CA were associated with the interface or the meeting point between 14q11 and 14q21. Thus, in this particular chromosome, all the potential CA were exclusively clustered in interbands. The

distribution of CA was found to be associated with the short arm of chromosome #15. Here all CA were mapped to band 15p12 although the non-randomness was not significant. A few CA were found in #16-#20 and X chromosomes. The χ^2-test revealed that the incidence of CA in these chromosomes was significantly lower than in other chromosomes ($p<0.005$). In other words, they were resistant to DMBA. A few were also encountered in Y chromosomes. Primarily, the interface between Yq11 and Yq12 was somehow involved preferentially in CA. All 5 CA were found in this interband. However, the distribution of CA in these regions was not significantly high on a statistical basis.

From the foregoing analyses, it became evident that many chromosomes were more or less primarily involved in CA induction to the chromosome-breaking proclivity of DMBA.

DNA replication pattern of rat chromosomes

The vulnerable regions in chromosomes #1 (1q31) and the two vulnerable regions in #2 (2q21 and 2q24) were shown to replicate DNA late in the S-phase although the 3rd susceptible 80% region in #2 did not reveal a late band by the present technique (Fig. 4). Other regions such as 3p13, 3p34, 4q32, 11p11, 11q11, 12p12, 13q13, 13p21, 19p14, and Yq11 were shown to replicate DNA in the late S-phase (Shown in Fig. 2 by arrows, redrawn from reference 25).

Effect of anemia, polycythemia, and EP on CA

In the anemic condition, the same distribution pattern was obtained although CA peaks became higher and more distinct than those in normal hemopoietic conditions, especially in chromosome #2. However, the peaks of CA in polycythemic rats were considerably suppressed, which was reversed by EP treatment (Fig. 1a-d). A detailed comparison of DMBA-induced CA in chromosome #2 of polycythemic rats with or without EP showed a prominent difference in the incidence of CA in the vulnerable regions. In contrast, the influence of EP was less prominent in the vulnerable regions of chromosome #1. Thus, the enhancing effect of EP on DMBA-induced CA was considered specific with respect to certain chromosomal regions.

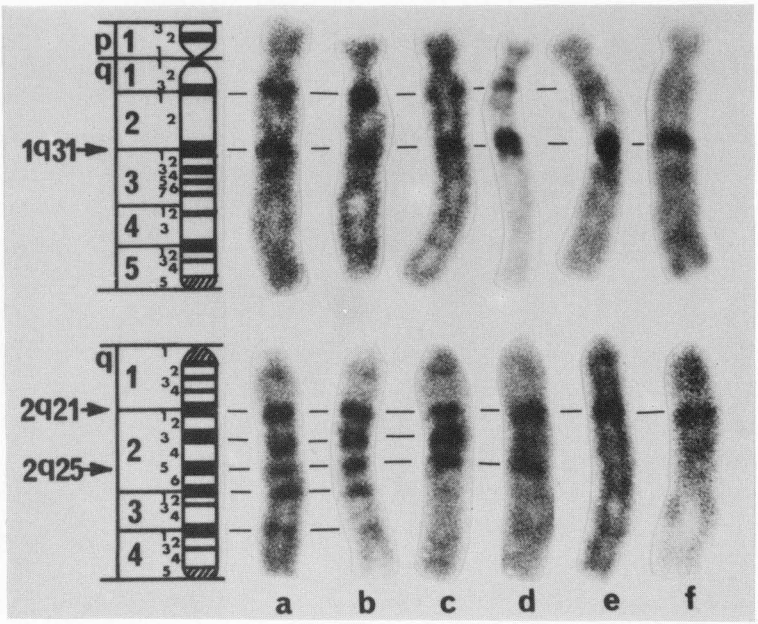

Fig. 4 DNA replication pattern of chromosomes #1 and #2 in late S-phase. a-c: dT-labeled for 4 hr. Most G-bands of chromosomes #1 and #2 are dT-labeled and gradually complete DNA replication in late S-phase. e-f: dT-labeled for 1.5 hr. Replication is concluded except in the dark-shaded regions.

DISCUSSION

With the introduction of banding techniques in the field of cytogenetics, the resolution of chromosome morphology has increased to a great extent. With the aid of this technique, CA could be significantly localized without ambiguity. In order to learn the sites of interaction of chemical carcinogens at the chromosomal level, locating break points along chromosomes was considered important. The present study was undertaken to reveal the specific break points for a chemical carcinogen to a particular chromosome band by measuring and the G- and Q-banding methods. A total of 427 DMBA-induced CA were mapped along Q-banded rat chromosomes. This is probably the first report so far carried out on rat chromosomes.

Non-random distribution of CA induced by chemicals, viruses

and radiation has been reported and this has been a subject of debate since the first report in this field by Sax[22] and Revell.[20] Although they first described non-random involvement in heterochromatic regions, later researchers indicated that CA were distributed variously, in R-bands,[5, 10, 15, 16, 19] G-bands or late-replicating heterochromatic regions,[3, 6, 8, 9, 17, 20, 28, 30] C-bands [5, 15, 16] G-R junctions,[3] and nucleolar or secondary constrictions.[8, 9, 11]

Following the report from our group on non-random distribution of DMBA-induced CA along #1 and #2 chromosomes of the rat,[28, 33] Popescu and DiPaolo[18] identified the specific break points on rat chromosomes following *in vitro* exposure to DMBA mainly in chromosome #1 and #2. They assessed the specific bands involved in CA by G-band method as 1q22, 1q43, 1q12, 1q34, 1q32, 1q36 and 1q37 in #1 and 2q22, 2q24, 2q26 and 2q34 in #2. All these broken regions were associated with the G-negative bands. With regard to frequent occurrence of CA, the largest telocentric chromosome (#2) was the most susceptible to the carcinogen action and amongst the negative band 2q24 collected the highest number of CA. The present studies also revealed that #2 chromosome was the most vulnerable and the Q-negative band, 2q24 had the highest number of CA. Two vulnerable regions were also identified in 1q22 and 1q43 of chromosome #1. These results were in completely accord with the results obtained by Popescu and DiPaolo.[18] The distribution of the break points was not confined to #1 and #2 chromosomes but extended to other chromosomes as shown Fig. 3.

The precise localisation of CA to band structure of individual chromosomes should be of great interest. The present study clearly showed that CA predominantly occurred in G-negative areas although some were also distributed in Q-positive bands or clustered around junctions between Q-negative and Q-positive bands. The predominant distribution of CA in the G-negative or euchromatic segment was in agreement with the results of many workers mentioned above. This preferential involvement of euchromatin might be due to the preferential interaction of early replicating DNA with clastogens. Similarly, occurrence of CA in Q-positive bands reported by Sugiyama et al.[32] reflects carcinogen interaction with late replicating DNA. The preferential occurrence of CA in a junction between heterochromatin and euchro-

matin has been reported mainly by Buckton[4] and Brøgger[3] in human chromosomes. In the present study, a significant number of CA associated with the interstitial bands were found in chromosomes #5, #14, #16 and #18 respectively, apart from Y chromosomes. The detection of an interface as a breakage site could be explained in the light of difference in chromosome structure at this particular region. This boundary region between dark and pale bands with different properties might be more fragile. The increased susceptibility of these bands is reported for damage induced by ionizing radiation,[7, 21] chemicals[15, 19, 21] and viruses[14] as well.

From the foregoing analyses, it became evident that the location of CA is in part dependent on the methods employed; Sugiyama[3] has shown that the distribution was dependent on time of harvest following carcinogen treatment. It also depends on the analysing method of break points. In other words, the break-points close to the borderline of neighboring Q-negative and Q-positive band, for example A, B, D, and E (indicated by arrows in Fig. 5), could be read as a break in the Q-negative band region microscopically. Therefore, if we postulate that CA are induced

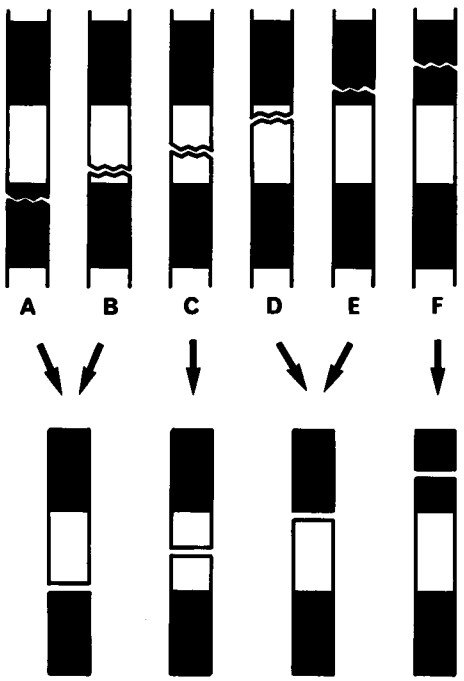

Fig. 5
The hypothetical relationship between actual breakage sites and apparent breakage sites. Breaks which occurred in interbands (C) and interface regions such as in A, B, D, and E appear to be breaks in interbands. Breaks in the mid-band regions (F) can be read as occurring in G-positive bands.

randomly, 5/6 of all CA would be assessed to be in Q-negative bands, and 1/6 in positive bands. This is supported by the fact that two peaks of CA along #2 chromosome were aplit respectively into two peaks in neighboring Q-negative bands, if CA were directly mapped on banded chromosomes. Buckton[4] mapped the break points using both R- and G-band technique to obtain a fair conclusion. Unlike many reports obtained by using G- or Q-band only, he showed that CA were mainly localized in interfaces. Therefore, the results obtained by G- or Q-band only may require further confirmation with R-band techniques.

On the other hand, certain susceptible regions such as 1q22, 2q16, 2q12, 2q24, 3q24, 4q34, 5q13, 6q24, 7q22, 11q12, 12p11, 14q21 have neighboring late replicating bands (Fig. 3). This fact may solve the gap between the present results and our previous results that some, if not all, late replicating heterochromatic G-positive regions are target sites for CA when chemical carcinogen was given a short time before cell-harvest or at least they influence the induction of CA in neighboring regions.

On the other hand, the present studies showed that functional chromosome structure such as EP-responsive sites may be also important in inducing CA. This may indicate for example that genes such as proto-oncogenes are target sites for CA since it is possible that these chromosomal sites are transcribed under growth stimuli and therefore become susceptible.

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