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Chromosome Structure as Revealed by a Combined Chemical and Immunochemical Procedure

(photooxidation/cytosine-specific antibodies/chromosome banding/immunofluorescence)

R. R. SCHRECK*, D. WARBURTON*, O. J. MILLER*†, S. M. BEISER‡, AND B. F. ERLANGER‡

* Department of Human Genetics and Development, † Department of Obstetrics and Gynecology, and ‡ Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

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ABSTRACT **Human metaphase chromosomes were photooxidized in the presence of methylene blue, a process that destroys guanine residues in DNA. Indirect immunofluorescence showed that such chromosomes reacted with a cytosine-specific antibody revealing a consistent fluorescent banding pattern by which each chromosome could be identified. The observed fluorescent patterns were the reverse of those produced in formamide-denatured chromosomes treated with an antibody specific for adenine and of the patterns obtained with quinacrine and with Giemsa staining by the G-banding techniques. The patterns were identical to Giemsa R-banding patterns. The chromosome banding patterns, therefore, appeared to reflect DNA base composition, indicating the feasibility of a combined chemical-immunochemical investigation of the chemical organization of chromosomes.**

It is possible to obtain anti-nucleoside antibodies (1) that are specific for the purine or pyrimidine determinant group of the immunizing antigen. By complement fixation (2) and radioimmunoassay (3), they show no crossreactions with any of the other bases present in nucleic acids. These antibodies react with single-stranded, denatured, or partially denatured DNA, but not with native DNA. They combine with fixed metaphase chromosomes, but only if the chromosomes are partially denatured (4). Using indirect immunofluorescence procedures, Dev *et al.* (5) found that anti-adenosine (anti-A) produced a characteristic pattern of light and dark bands along human metaphase chromosomes that had been treated for 1 hr at 65° with 95% formamide in the presence of 0.25% formaldehyde. The pattern obtained corresponded closely with those produced by quinacrine or by the Giemsa G-banding techniques (6–8), a finding consistent with the evidence that enhanced fluorescence of quinacrine occurred in AT-rich regions of DNA (9–11). The results suggest, moreover, that the specificity of anti-nucleoside antibodies observed in solution is carried over to the chromosome system.

If anti-nucleoside antisera are indeed as specific in chromosomal preparations as they are in solution, it should be possible to map chromosomes chemically by use of various chemical and immunochemical procedures in combination. The more selective the chemical procedure, the more information one should be able to obtain with anti-nucleoside antibodies. Simon and Van Vunakis (12) have shown that guanine residues are selectively destroyed by photooxidation of DNA in the presence of methylene blue. No breakdown of the other DNA bases was observed. Their results were confirmed by Garro *et al.* (2), who also demonstrated that the destruction of guanine exposed cytosine residues that could be detected by cytosine-specific antibody (anti-C). In this paper, we describe experiments in which chromosome preparations were photooxidized in the presence of methylene blue and then treated with anti-C. The fluorescent banding patterns produced were the reverse of quinacrine and of anti-A patterns and, therefore, were consistent with the known specificities of the chemical and immunochemical reactions. The results suggest that the banding produced by quinacrine and by the nucleoside-specific antibodies are a reflection of the base composition of chromosomes.

Abbreviations: anti-A and anti-C, adenosine- and cytosine-specific antibodies, respectively; PBS, phosphate-buffered saline pH 7.2–7.4.

MATERIALS AND METHODS

Chromosome preparations were made from human leukocyte cultures by the method described by Dev *et al.* (5). The slides were air-dried to eliminate any possibility of heat denaturation.

The slides were photooxidized in a solution containing 33.4 μM methylene blue (Allied Chemical-National Aniline Division) in 0.1 M Tris·HCl buffer (pH 8.75). One to three slides were placed in a coplin jar with 50 ml of the dye solution, and oxygen was bubbled through the solution for 10 min. The jar was then quickly and tightly sealed and was placed in a glass, temperature-controlled, water bath (25°) at a distance of 15 cm from a 150-W Sylvania flood lamp. The illumination of the jar at this distance was about 64,600 lux, as measured by a Wescott light meter. The slides were exposed to light overnight (15–18 hr). The final temperature within the coplin jar was 26°, and the dye solution was noticeably lighter in color at the end of the experiment.

Slides were then rinsed briefly in PBS [phosphate-buffered saline: 20 g of NaCl–85 ml of 0.25 M Na_2HPO_4–15 ml of KH_2PO_4 in 2400 ml of distilled water pH 7.2–7.4)]. They were layered with anti-C [prepared in rabbits as described by Erlanger and Beiser (1)], which was diluted 1:10 with PBS and left in a humid chamber at room temperature (25°) for 30 min. They were rinsed with a spray of 200 ml of PBS, then layered with fluorescein-labeled antibody, prepared in sheep, against rabbit immunoglobulin G, which was diluted 1:50 with PBS, and incubated and rinsed as before. A coverslip was wet-mounted with PBS. Some photooxidized slides were not treated with the antibodies but were stained with quinacrine mustard. All slides were observed with a Zeiss fluorescent microscope fitted with an HBO 200-W mercury

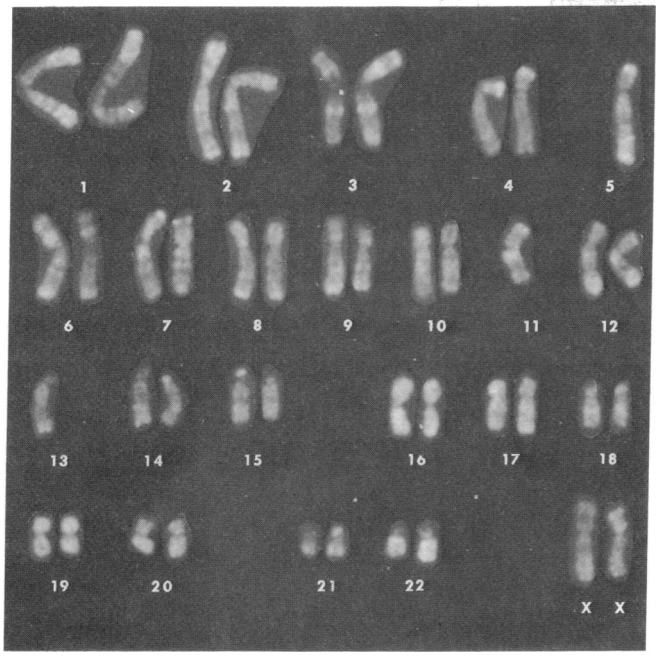

FIG. 1. The human karyotype produced by anti-C after photooxidation.

lamp, a BG12 exciter filter, a 530-nm barrier filter, and a 100X Planapochromatic objective. Well-spread fluorescent metaphases were photographed on Panatomic X or H & W control film with exposure times of about 2 min. Antibody-treated cells could be photographed only once because the fluorescence faded rapidly. A control slide left in oxygenated methylene blue, but not exposed to light, was treated with anti-C and showed no antibody uptake. Other control slides were treated with anti-A or anti-thymine after photooxidation.

RESULTS

After photooxidation and indirect immunofluorescence with anti-C, a consistent pattern of light and dark bands was observed in human chromosomes. Each chromosome could be identified by its characteristic banding pattern, and karyotypes of eight cells were prepared (Fig. 1). In the 50 cells observed, the banding patterns were the reverse of those produced by quinacrine or by anti-A (Figs. 1 and 2). There was minimal uptake of anti-C in the regions previously shown to react most strongly with anti-A. Fig. 2 compares patterns obtained by several techniques. In chromosome 1, the distal end of the short arm, which is pale with quinacrine, was extremely bright when treated with anti-C after photooxidation. The centers of the long arms of chromosomes 11 and 12, which are bright with quinacrine, appeared dull after treatment with anti-C. The distal portion of the Y chromosome, which is intensely fluorescent with quinacrine, was extremely dull with anti-C.

Photooxidized chromosomes, not treated with antibody, showed a normal quinacrine banding pattern. Slides treated with anti-adenosine or anti-thymine after photooxidation showed a pale fluorescence over the chromosomes, but gave no evidence of banding.

Slides prepared from an Indian muntjac (*Muntiacus muntjac*) fibroblastic cell line were also photooxidized and

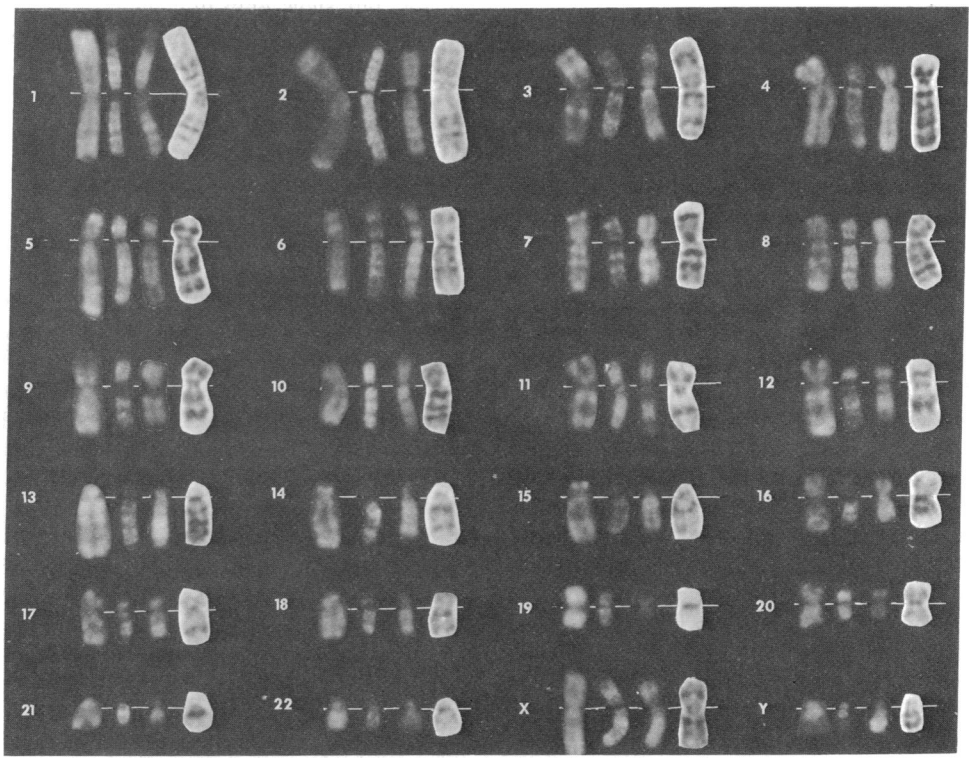

FIG. 2. The 24 human chromosomes as seen by anti-C, anti-A, quinacrine, and Giemsa G-banding techniques (*left* to *right*). Note the close correspondence of the banding patterns produced by the last three techniques. The banding patterns produced by the anti-C technique are the reverse of the other three, with intense staining of bands that are only lightly stained by anti-A, quinacrine, or Giemsa G-banding methods.

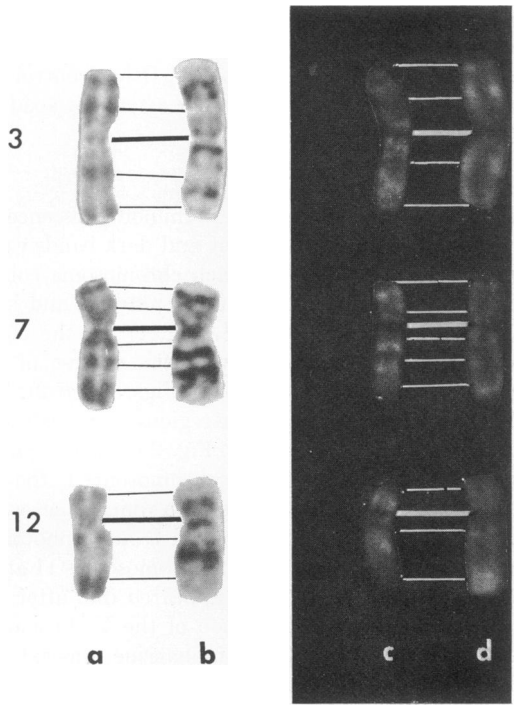

FIG. 3. Human chromosomes 3, 7, and 12 showing the reciprocal banding patterns produced by the following techniques: (*a*) R-banding compared to (*b*) G-banding, and (*c*) anti-A banding compared to (*d*) anti-C banding. *Broad lines* connect centromeres. A *narrow line* connects each of the more intensely stained bands of the chromosomes in columns *a* and *d* to the corresponding poorly stained band in columns *b* and *c*, respectively. Note the close correspondence of the Giemsa R-banding with the anti-C patterns, and the Giemsa G-banding with the anti-A patterns.

treated with anti-C. Although optimal conditions for their photooxidation have not yet been determined, areas of intense fluorescence could be observed. The pattern was the reverse of that produced by quinacrine. Particularly notable was the small Y chromosome, which was quite pale with quinacrine and Giemsa banding, but extremely bright after treatment with anti-C.

DISCUSSION

Our results provide strong support for the hypothesis that chromosome banding patterns reflect localized AT-rich and GC-rich regions in DNA. The anti-cytosine sera used in these experiments had been thoroughly characterized immunochemically and shown to be reactive with denatured DNA, and to be highly specific for cytosine. Similar serum had been used to detect cytosine residues exposed by photooxidative destruction of guanine in DNA (2). It can be concluded, therefore, that photooxidized chromosomal regions that fluoresced because they bound anti-C were originally rich in GC pairs. These regions were generally the ones that fluoresced least after quinacrine staining or after reaction with anti-A. The findings are consistent with the belief that quinacrine fluorescence is enhanced in AT-rich regions of DNA and quenched in GC-rich regions (9–11). Further support stems from our demonstration of normal quinacrine banding after photooxidation. Quinacrine intercalation would be expected only in chromosomal regions that retained an intact structure after photooxidation, i.e., AT-rich regions. The suggestion that quinacrine mustard produces banding by selectively binding to guanine (13), therefore, appears unlikely.

There is another chromosome banding technique that produces a pattern that is the reverse of quinacrine or standard Giemsa G-banding, and corresponds closely to that seen after our anti-C treatment. This is the "controlled heat denaturation" or R-banding (14) method of Dutrillaux and Lejeune, in which chromosome preparations are heated in balanced salt solution (pH 6.5) at 87° for 10–20 min and then stained with Giemsa (15, 16). The resulting pattern of light and dark bands is complementary to that seen with the other Giemsa banding methods, which use pretreatment with proteolytic enzymes, warm saline solutions, or other agents. Thus, while G-banding gives basically the same pattern obtained with anti-A, R-banding is identical with anti-C banding (Fig. 3). The existence of at least two techniques that give complementary banding patterns suggests that a simple nonhomogeneous distribution of DNA or total mass before staining is not the explanation for chromosome banding patterns. Similarly, Caspersson *et al.* (17) have shown that the quinacrine fluorescent banding patterns of chromosomes do not parallel the DNA content along the chromosomes as measured by UV-absorption spectrometry.

The heterochromatic chromosomal regions are apparent exceptions to the otherwise consistent relationship between quinacrine, anti-A, and anti-C banding patterns. The distal part of the human Y chromosome is brilliant after quinacrine staining, and, as expected, negative to anti-C after photooxidation. However, it fails to react with anti-A after formamide treatment, indicating that this region may be highly resistant to denaturation. The secondary constrictions of chromosome 1, 9, and 16 are negative to quinacrine and anti-A, but show only dull fluorescence when reacted with anti-C. Perhaps the protein structure of these regions makes them resistant to photooxidation. Weil *et al.* (18) have shown that histidine, methionine, tryptophan, tyrosine, and cystine were photooxidized to different degrees under conditions similar to those used in our studies. These amino acids are present in the so-called acidic residual proteins, and to a lesser extent in the other class of chromosomal structural proteins, the basic histones. There is evidence that the distribution of nucleoproteins is related to the base composition of DNA in localized areas of chromosomes (19–21). Thus, protein might influence the banding patterns by screening certain specific areas from photochemical effects.

It is perhaps surprising that such large foci of AT- or GC-rich DNA would be present throughout the length of each chromosome. The human haploid complement at metaphase contains only about 320 bands demonstrable by quinacrine or Giemsa staining (14). Many bands, therefore, must contain several hundred genes of unique DNA sequence. Such large collections of informational DNA would be extremely unlikely to differ greatly from one another in their average base composition. However, a significant proportion of the DNA of mammalian genomes may be composed of repetitious DNA (22–24), which can vary greatly from the average base ratio. We, therefore, suggest that the organization of the large amounts of repetitive DNA base sequences in metaphase chromosomes permits neighboring chromosome segments to have wide differences in their average base composition and that chromosome banding reflects these differences. A similar model was recently proposed by Sutton (25). It should be

mentioned at this point that neither the present work nor earlier work done in these laboratories (3–5, 24) support Crick's suggestion (22) of the presence of single-stranded DNA in chromosomes during any phase of cell development other than the S phase, a time when DNA is replicating.

Since the anti-nucleoside antibodies appear to be as specific in chromosome preparations as they are in solution, we can anticipate at least modest success in future attempts to map chromosomes chemically by using a variety of combined chemical and immunochemical procedures. We have coupled one specific chemical reaction, the photooxidation of guanine, with the use of anti-cytosine to localize GC-rich regions of chromosomes. A chemical denaturation procedure involving formamide has been used with anti-adenosine to locate AT-rich regions of chromosomes. The use of various antisera and specific chemical techniques will provide a means of relating the chemical composition of normal and abnormal chromosomes to their structure as revealed by both light and electron microscopy. Similarly, such procedures might lead to further understanding of such problems as the effects of mutagenic agents on chromosomal DNA.

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