

Specific Banding Patterns of Human Chromosomes

(heterochromatin/Giemsa stain/chromosome bands)

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ABSTRACT Individual pairs of human chromosomes can be reliably identified by a new method that does not require special optical equipment and that results in permanent preparations. This method, which is based on treatment of the chromosomes *in situ* with NaOH, followed by incubation in sodium chloride-trisodium citrate and Giemsa staining, results in highly specific banding patterns in characteristic regions of the chromosome arms. It should prove useful for the detection of small structural changes in chromosomes.

During the past 15 years remarkable progress has been made in the descriptive morphology of both normal and abnormal human chromosomes, but with conventional cytological and autoradiographic techniques we have thus far failed to identify every chromosome in the human complement. Furthermore, these techniques have revealed very little differentiation within a chromosome arm, so that structural rearrangements that do not alter the length or centromere position, or produce only slight changes in these, will escape detection.

Since the original description of specific fluorescent staining of human chromosomes (1), investigations have led to renewed hope for subdividing the classical chromosome groups into individual pairs. This paper describes a new method of differentiating human chromosomes that does not require fluorescence microscopy and that results in permanent preparations. The chromosomes exhibit banding patterns in specific regions. In addition to the sex chromosomes, 20 of the 22 autosomal pairs have been identified by this technique, and the remaining two are tentatively classified.

MATERIALS AND METHODS

Lymphocyte and fibroblast cultures from normal persons were prepared and harvested in the usual manner. Colchicine, 0.04 $\mu\text{g}/\text{ml}$ of medium, was added to the cultures 2 and 6 hr before harvest. After hypotonic treatment with 1% sodium citrate and fixation in methanol-acetic acid 3:1, flame-dried slides were prepared.

The method to be described consists of treatment of the chromosomes *in situ* with NaOH, followed by incubation in several concentrations of a saline-citrate solution (SSC). Specific regions of the chromosome arms then stain differentially with buffered Giemsa. Because a number of parameters have varied in our pilot experiments and we do not yet know the optimal conditions for treatment, we will present first a basic procedure that we have found to produce bands fairly consistently, and then a range of times and concentrations that have been tested.

The slides are treated for 30 sec in a solution of 0.07 N NaOH in 0.112 M NaCl (pH 12.0) at room temperature and then rinsed three times in 12 \times SSC (pH 7.0) for 5-10 min each time. They are then incubated in 12 \times SSC at 65°C for 60-72 hr. After incubation, the slides are passed through three changes of 70% ethanol and three changes of 95% ethanol (3 min each). After air-drying, slides are stained for 5 min in buffered Giemsa solution (pH 6.6), rinsed briefly in distilled water, air-dried again, and mounted in Permount.

The alkaline solution is prepared by adding 2.8 g of NaOH and 6.2 g of NaCl to 1 liter of distilled water. The 12 \times SSC solution is made with 105.2 g of NaCl and 52.9 g of trisodium citrate in 1 liter of distilled water, adjusted to pH 7.0 with 0.1 N HCl. The buffered Giemsa solution consists of: 5 ml of Giemsa stock solution (Curtin Scientific Co.), 3 ml of absolute methanol, 3 ml of 0.1 M citric acid, and distilled water to 100 ml, adjusted to pH 6.6 with 0.2 M Na_2HPO_4 .

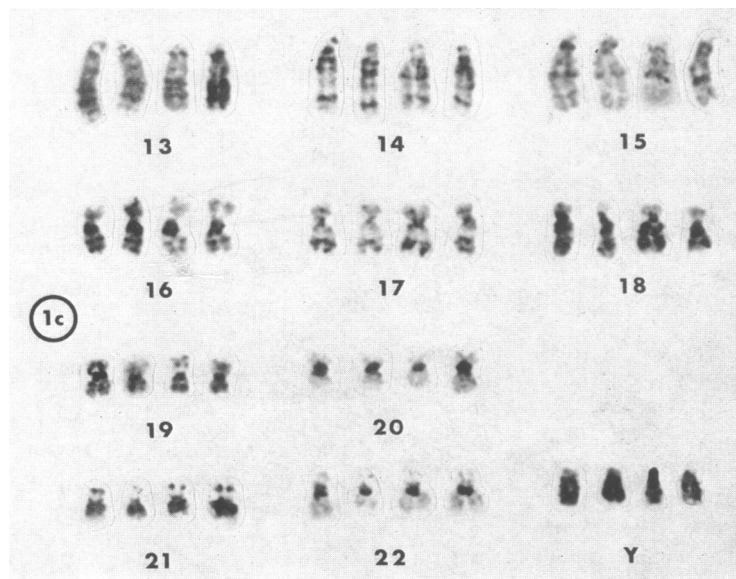
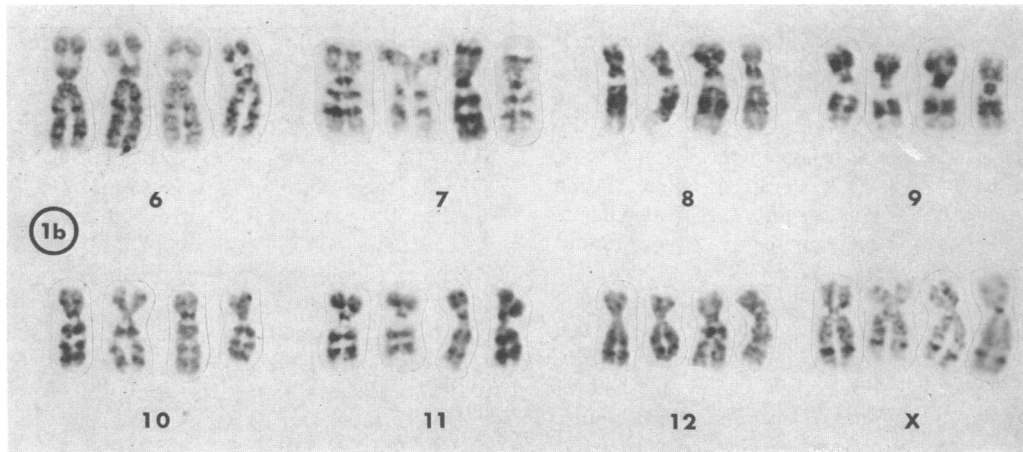
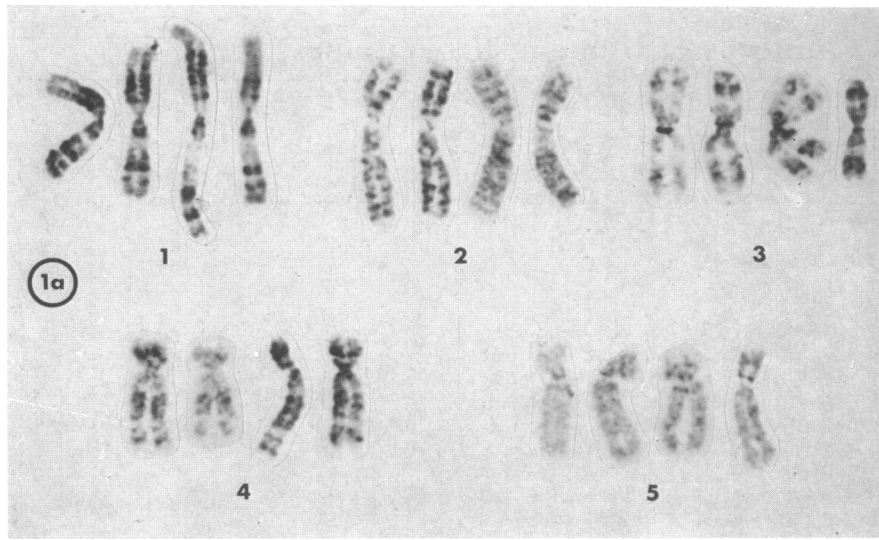
The method described here produces discrete, sharp bands in many of the metaphase cells; the bands are always in the same sites, characteristic for a particular chromosome. The following methods have also been used: (a) NaOH treatment at 0°C, and room temperature for 0-180 sec; (b) Incubation in 2 \times , 6 \times , 12 \times , and 24 \times SSC for various intervals from 15 min to 136 hr; (c) Giemsa stain from 5 to 90 min, with or without prior treatment of the chromosomes; (d) incubation in 1:1 formamide-12 \times SSC at 37°C for 1-5 days.

Although some combinations of the above conditions occasionally produced informative results, there was often merely a suggestion of indistinct banding patterns. An uncoiling effect, rather than distinct bands, was sometimes observed. Excessive NaOH treatment (longer than 1 min) resulted in pale and swollen chromosomes with an "empty" appearance. Other metaphase cells revealed compact, heavily-stained, normal appearing chromosomes. The "informative" cells appeared much more frequently when the method first described above was used. Only cells showing well-spread chromosomes with differentially stained bands were selected and photographed.

Fifteen selected cells from three male individuals were photographed and karyotyped without regard to banding patterns. The photographs of the chromosomes were classified into the following groups: A1, A2, A3, B, C, D, E, F, and G + Y. Photographs of distorted, swollen, and overlapping chromosomes were discarded and the remainder were labeled on the back by cell number and group.

The chromosome photographs of all 15 cells were then pooled by group. At this point, without knowing the identity of the cell number, we subclassified each group according to

Abbreviation: SSC, 0.15 M NaCl-0.015 M sodium citrate.



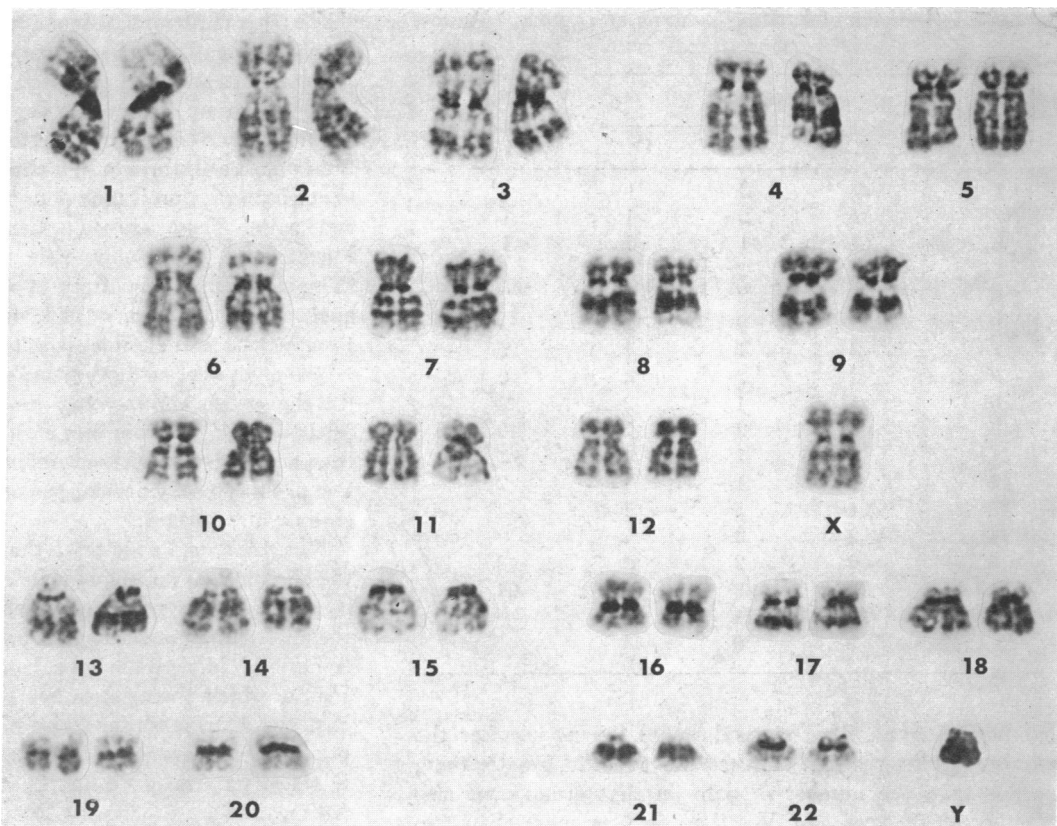


FIG. 2.

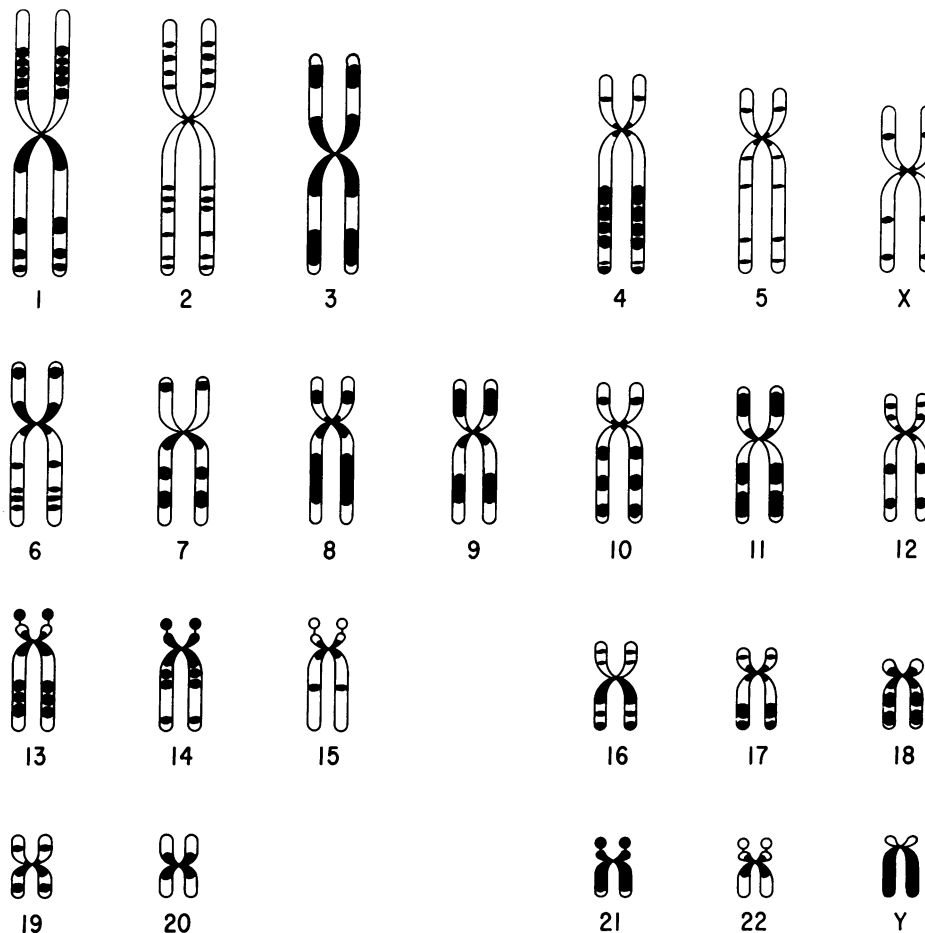


FIG. 1a-c. Examples of banding patterns of human chromosomes. In each set of four, the first two are homologs from one cell, while the third and fourth are examples from two other cells.

FIG. 2. Complete karyotype of endoreduplicated cell exhibiting banding patterns in diplochromosomes.

FIG. 3. Diagram of most reliable patterns for each chromosomal pair. Within groups, the numbering system is arbitrary except for A1, A2, A3, C6, E16, and Y.

FIG. 3.

TABLE 1. Analysis of banding patterns of D-group chromosomes among 15 male cells

Cell no.	Pattern			Unidentified or missing
	D13	D14	D15	
1	0	3	1	2
2	2	2	1	1
3	2	1	2	1
4	2	2	1	1
5	1	2	1	2
6	1	0	2	3
7	2	1	1	2
8	2	2	2	0
9	1	1	2	2
10	2	2	2	0
11	2	1	1	2
12	2	1	1	2
13	1	1	1	3
14	2	2	2	0
15	0	2	1	3
Total	22	23	21	24
Definite	13	14	10	
Probable	9	9	11	

banding patterns. This method would tell us whether the number of patterns that emerged was equal to, greater than, or less than the number of pairs in that group, and also whether there were two, less than two, or more than two chromosomes assigned to a specific pattern in each cell.

Complete karyotypes of these 15 cells and other cells from both male and female blood donors were analyzed by this method to determine the reliability and usefulness of the prototype idiogram of bands for individual pair identification.

RESULTS

A detailed analysis of the D13-15 group will first be illustrated. The other groups were analyzed in a similar manner. Banding patterns for all of the chromosome pairs are shown in Figs. 1 and 2.

Among 90 D-chromosomes in the 15 cells, three could not be morphologically identified because they were missing, distorted, or overlapping. Among the 87 remaining D-chromosomes, 21 were uninformative as to banding pattern, because they appeared swollen and unbanded or because the banding was diffuse rather than discrete.

Three banding patterns were immediately evident. Each of the 87 D-chromosomes was classified into one of the three patterns as "definite", "probable", or "unidentifiable". These patterns were arbitrarily labeled D13, D14, and D15 (Fig. 1c and Table 1). Pattern D13 was characterized by a wide, multi-banded region in the distal two-thirds of the long arm. Pattern D14 had a concentration of several bands in the proximal one-third of the long arm and a single discrete band near the end of the long arm. Pattern D15 showed only light nondescript banding with the exception of a single, fine, dark band midway down the long arm.

Table 1 shows that there were 22, 23, and 21 chromosomes assigned to the three patterns. In three cells (nos. 8, 10, and 14), all six D-chromosomes were grouped into three pairs. In only one instance (no. 1) did three chromosomes emerge as one pattern; in this case one of them was classified as "prob-

able". This chromosome was overstained and our guess was simply wrong. Table 1 also shows that over 75% of the D-chromosomes analyzed exhibited specific banding patterns.

The C group deserves special comment. At first, only seven patterns emerged (theoretically there should be eight). But in most cells three or four chromosomes had been assigned to one pattern; thus it is obvious that we are dealing here with two pairs. These are tentatively labeled C11 and C12. Furthermore, since only male cells were analyzed above, there should be one pattern in which only one chromosome appears, the X. This, in fact, occurred. In only one of the 15 cells were two chromosomes assigned to this pattern.

The analytical method described above was used to establish the specific chromosomal landmarks that are now being used to karyotype complete cells from other donors. This has been accomplished with several other healthy individuals and two phenotypically normal persons who bear a morphological chromosome variant.

Figs. 1a, b, and c illustrate the banding patterns of each of the chromosomes of the human complement. Fig. 2 shows a complete karyotype of an endoreduplicated cell. Note that the diplochromosomes match for specific banding patterns. A stylized idiogram of the banding patterns is shown in Fig. 3, which summarizes our principal observations. Note that the distribution and size of the lightly stained "inter-band" regions are useful landmarks. This is most prominent in the short arm of C6 and both mid-arms of A3, but other light regions are also informative.

The specificity of the patterns illustrated in Figs. 1-3 is supported by the correlation of similar banding patterns in: sister chromatids of the same chromosome, homologous chromosomes within each cell, cells within the same individual, cells among different individuals, diplochromosomes in endoreduplicated cells, and chromosomes in polyploid cells.

DISCUSSION

It is evident that the technique described induces the appearance of discrete and specific chromosome banding patterns. Although we do not understand the molecular mechanisms that produce these bands, some hypotheses can be suggested.

The basic technique is a modification of the procedure of Pardue and Gall (2), and reported by Arrighi and Hsu (3), for the differential staining of constitutive heterochromatin of human chromosomes. Arrighi and Hsu (3) used HCl treatment to remove histones and other nonacidic proteins, RNase to remove chromosome-associated RNA, then NaOH treatment of the chromosomes *in situ* for 2 min and incubation for 18-24 hr in 2× or 6× SSC at 65°C. The darkly-stained heterochromatin blocks revealed by the Giemsa stain after this treatment appear in the following regions: small blocks in the paracentric regions of all chromosomes (proximal heterochromatin or centric heterochromatin); larger blocks in the proximal long arms of chromosome pairs A1, C9, and E16 (secondary constriction regions); and a very prominent block in the distal two-thirds of the long arm of the Y chromosome (Y heterochromatin).

For ease of discussion, we shall refer to the method of Arrighi and Hsu (3) as the "block" technique (since blocks of darkly-staining heterochromatin appear), and to the method described in this paper as the "band" technique. There are both positive and negative correlations between these two methods.

(a) The centric heterochromatin is much less conspicuous by the "band" technique.

(b) The secondary constrictions of A1 and E16 are positively stained by both techniques but there is an inverse correlation for C9. The secondary constriction of C9 is very heavily stained by the "block" technique, but is virtually unstained by the "band" technique.

(c) The Y heterochromatin is the most positively stained region of human chromosomes by both methods but, in addition, the proximal one-third of the long arm and sometimes the short arm of the Y are also heavily stained by the "band" technique, whereas these regions are usually negative by the "block" method.

(d) Comparison of male and female cells showed that the X chromosome is not identifiable by the "block" method. The X banding pattern shown in Fig. 2 was present in only one C chromosome in the male but in two C chromosomes in the female. Thus, the facultative heterochromatin in the late-replicating X chromosome in the female does not respond differently to the "band" technique.

As the block method is based on a denaturation-renaturation process (3), it is tempting to speculate that the centric heterochromatin that is demonstrated by the method is the rapidly annealing, highly repetitive DNA (4), while the bands scattered throughout the genome represent families of repeated sequences with fewer copies (5, 6). The unstained interband regions could then be the sites of unique nucleotide sequences. This hypothesis is supported by the fact that a longer incubation period is required to reveal the bands.

We have found that $12\times$ SSC was more effective in producing bands than $2\times$ SSC, $6\times$ SSC, or $24\times$ SSC. Incubation periods beyond critical limits or incubation with no prior NaOH treatment showed only a "suggestion" of very weak bands or no effect at all. Moreover, during different periods of incubation we were able to follow a sequence of changes. Before the appearance of bands in the chromosomes, an "uncoiling" appears, and after longer periods of incubation, the chromonemata appear more discrete, while in certain cases chromosomes with a lampbrush-like effect are produced. This would suggest that in the process of producing a banded appearance, changes in the arrangement of coils are involved. In this respect, Shiraishi (7) has recently reported that human leukocyte chromosomes show differential reactivities after treatment at low temperature ($0-3^{\circ}\text{C}$) during the last 24 hr of culture. Prominent segments or coils appeared along the chromosome arms in regular sequence, thus distinguishing individual pairs. It is not known whether any of these segments are correlated with the heterochromatin banding patterns described above.

The Giemsa stain is of utmost importance. Both the quality of the stain and the length of staining time are critical, since overstaining will obscure the banded appearance. The Giemsa stain is probably reacting in a very specific way with

the chromosomes. Bands are not visible in treated slides that are stained with aceto-orcein.

Specific affinity of certain chemicals to chromosome segments has been demonstrated by Caspersson *et al.* (1) by the fluorescence technique. Apparently the heterochromatin bands are rather well correlated with the fluorescent bands produced with quinacrine mustard (8), with a few exceptions. Several comments are appropriate: (a) The centric heterochromatin region and the secondary constrictions of A1, C9, and E16 do not fluoresce as much as the arms of the chromosomes. (b) The Y heterochromatin is very brightly fluorescent and also stains heavily by both "band" and "block" techniques. (c) The heavily fluorescent band near the centromere of A3 is not stained by either the "band" or "block" heterochromatin technique. (d) The mid-region of the short arm of C6 is negative to both fluorescence and banding.

Individuals with chromosomal structural rearrangements may now be examined for identification of the chromosome(s) involved. In the cells from one phenotypically normal person, a D-chromosome with a giant satellite was identified as D13 by the banding pattern. Thus, the banding method holds promise for further delineating and identifying chromosomal variation in both healthy and diseased states. This method is simple, inexpensive, and easily adapted to routine laboratory use. It should be possible to refine the analysis and quantitate the results more precisely by computer scanning procedures and microspectrophotometry (9).

Another application of the banding method is the comparative analysis of banding patterns in various species and subspecies similar to that described by Arrighi *et al.* (10) for the block technique. We have already observed distinct banding patterns in synchronized Chinese hamster cells.

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