Proc. Natl. Acad. Sci. USA
Vol. 74, No. 3, pp. 1245–1249, March 1977
Medical Sciences

Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants

(mutants/sex determination)

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Communicated by V. A. McKusick, December 14, 1976

ABSTRACT **A number of individuals with aberrant Y chromosomes have been tested for the presence of Y-chromosome-specific reiterated DNA. These studies locate Y-chromosome-specific reiterated sequences on the long arm of the Y chromosome. Correlation with phenotype and other known Y chromosome markers establish that the Y-chromosome-specific reiterated DNA discussed here has no evident role in male determination.**

Among all the examples of genetic regulation in mammals, the essential role of the Y chromosome in primary sex determination is possibly the best known but the least well understood (1, 2). Until recently, there existed no genetic markers for the Y chromosome and thus no way to correlate chromosome structure with male function. This situation is now changing with the discovery of two distinctive cytological characteristics on the human Y chromosome (3, 4) and the recognition that a locus for a male histocompatibility antigen is Y-linked (5, 6).

We have recently identified reiterated DNA sequences specific for the human Y chromosome (7). These sequences represent at least 10% of Y chromosome DNA. They are used here for the initial stages of a correlative structure-function study of the human Y chromosome. We show that this particular group of human Y-chromosome-specific reiterated DNA is probably limited to the long arm of that chromosome. While we establish that these sequences play no evident role in dictating maleness, our approach—when adapted to other Y chromosome sequences—may lead not only to a further understanding of the molecular origins of sexual differences, but also to a test of the putative regulatory function (8, 9) of reiterated DNA.

MATERIALS AND METHODS

Subjects. Blood was obtained in accord with the principle of informed consent from normal 46,XY males, normal 46,XX females, and 10 individuals with aberrant karyotypes. Variant subjects included:

Individual 1. A prepubertal phenotypic female with gonadal streaks and no evidence of testicular elements. Chromosomal analysis, including quinacrine fluorescence, showed a 46,X,i(Yq) karyotype, i.e., apparent lack of Y chromosome short arm and a doubling of the Y chromosome long arm (J. M. Rary and H. W. Jones, unpublished data). Although the i(Yq) chromosome seems to have only one centromere, the presence of a second centromere and a small amount of short arm material cannot be excluded.

Individual 2. A tall, phenotypically normal male of above average intelligence with a 47,XYY karyotype (10).

Individual 3. A previously described (11) physically normal male, who has a 46,XYq− karyotype and lacks the brightly quinacrine-fluorescent distal segment of the Y chromosome, i.e., approximately half of the long arm of this chromosome.

Individual 4. A male paternal fifth cousin of individual 3, who has a similar karyotype.

Individual 5. A 15-year-old, phenotypic female who had been hospitalized for removal of an abdominal Sertoli cell tumor (12). Karyotypes of both gonadal streak and skin fibroblasts as well as peripheral blood lymphocytes all showed a Y/autosome translocation. A more recent study, which utilized quinacrine fluorescence, showed mosaicism with two equally abundant cell types and a karyotype of mos45,X,t(Y;17)(Yqter → Yq11::17p13 → 17qter)/46,X,t(Y;17)(Yqter → Yq11::17p13 → 17qter; 17pter → 17p13::Yq11 → Ypter), i.e., one half the cells contained a translocation of the long arm of Y and the other half contained a very small chromosome, possibly the reciprocal short arm translocation, as well as the long arm translocation.

Individual 6. A previously described (13) female with some stigmata of Turner's syndrome. Quinacrine fluorescence showed a 46,X,t(X;Y)(:Xq22 → Xp11 or Xpter → Xq11::Yq11 → Yqter) karyotype. Analysis of buccal smears for Barr body coupled with quinacrine fluorescence on the same slides suggested that the inactive X chromosome was the translocation chromosome. Late labeling (14) of this chromosome with BrdUrd supported this conclusion. In this translocation chromosome, the distance between the terminus of the Y long arm and centromere was greater than usually encountered in the Yq of normal individuals. We therefore suppose that the translocation chromosome centromere is derived from the X chromosome and that the Y centromere and short arm are absent.

Individual 7. A normal female with a 46,XX,15p+22p+ karyotype, who is a mother of a normal son and a member of the same Pennsylvania Amish community in which individuals 3 and 4 reside (D. S. Borgaonkar, unpublished data). The intensity of quinacrine-fluorescent satellites on one chromosome 15 and one chromosome 22 resembled that normally associated with the distal segment of Yq. This raised the possibility that the novel satellites might represent translocants from Yq.

Individual 8. A phenotypic male with small testes, short stature, and mental retardation, with a mos45,X/45,X,t(Y;22)(Yp11 → Ypter or Yq11 → ?::22pter → 22q13) karyotype

Abbreviations: C_0t, molar concentration of DNA nucleotide times the duration of incubation in seconds; it-Y DNA, human Y-chromosome-specific reiterated DNA; 1 A_{260} unit, that amount of material which when dissolved in 1 ml of solvent gives an absorbance of 1 with a pathlength of 1 cm.

(15). The chromosome segment translocated to the long arm of 22 occurred in approximately 66% of cells. Although the origin of this segment is unclear, its size and lack of quinacrine fluorescence makes it resemble either the short arm of the Y or a proximal portion of the long arm of the Y.

Individual 9. A 25-year-old male with Kallman's syndrome (hypogonadotropic hypogonadism, anosmia) (16) and a 46,XX karyotype. No sign of Y chromosome was revealed by quinacrine fluorescence. Barr bodies were present in buccal smear preparations (D. S. Borgaonkar, unpublished data).

Individual 10. A 23-year-old male with normal testes, short stature, and severe mental retardation, with a mos45,X/46,X?Y karyotype (W. R. Breg, unpublished data). Sixty-six percent of the cells showed a Y-like chromosome which lacked the quinacrine fluorescence distinctive of a normal Y long arm.

DNA Isolation. Leukocyte DNA was isolated from 50 to 100 ml of whole blood that had been collected in acid citrate dextrose or sodium heparin. One volume of whole blood was diluted with 9 volumes of cold buffer (0.32 M sucrose, 5 mM $MgCl_2$, 1% Triton X-100, and 0.01 M Tris·HCl at pH 7.6 at 25°) and the mixture homogenized at 4° in a glass vessel with a motor-driven Teflon pestle. After centrifugation (2500 × *g*, 15 min) at 4°, the nuclear pellet was resuspended in 25 ml of cold 75 mM NaCl, 24 mM NaEDTA, pH 8.0, and disrupted by the addition of 1.25 ml of 20% sodium dodecyl sulfate. The mixture was incubated for 2–4 hr at 37° with Pronase (100 μg/ml, Calbiochem) and extracted with phenol and then with chloroform. DNA was precipitated by the addition of 50 ml of 95% ethanol and spooled onto a glass rod. The ethanol precipitate was gently resuspended in 20 ml of a 15 mM NaCl, 1.5 mM sodium citrate solution and stored over chloroform. Yields of DNA ranged between 30 and 50 μg/ml of whole blood.

Preparation of Radiolabeled Y-Chromosome-Specific Reiterated DNA. Tritiated Y-chromosome-specific reiterated DNA (it-Y [^{3}H]DNA) was prepared as outlined previously (7). In brief, sonicated DNA isolated from a 47,XYY individual was first fractionated on the basis of reiteration frequency and reassociated duplex stability. The most stable reassociated reiterated duplexes were radiolabeled by the nick translation function of DNA polymerase I (17). Radiolabeled sequences (about 7.5 × 10^7 dpm/μg), representing 3% of the genome, were mixed with a 40,000-fold excess of unlabeled and unfractionated DNA isolated from a 46,XX female; the mixture was heated to 100° and then incubated at 60° to a C_0t of 460. (C_0t is the molar concentration of DNA nucleotide times the duration of incubation in seconds.) Nonreassociated radiolabeled molecules were collected from hydroxylapatite and once again challenged with a large excess of 46,XX DNA. Those radiolabeled sequences that twice failed to reassociate to 46,XX DNA were specific for the Y chromosome (7). After purification, the mean piece size of it-Y [^{3}H]DNA was approximately 180 nucleotides. It should be noted that it-Y [^{3}H]DNA prepared in this manner contains not only radiolabeled sequences specific for the Y chromosome, but also degraded radiolabeled sequences incapable of reassociation as well as unlabeled single-copy sequences.

Reassociation Assays. DNA isolated from each individual was assayed, in excess, by reassociation with trace amounts of it-Y [^{3}H]DNA (7). After sonication to a mean piece size of 800 nucleotides, 25 A_{260} units of each DNA to be tested were mixed with 12,000 dpm of it-Y [^{3}H]DNA. (1 A_{260} unit is that amount of material which when dissolved in 1 ml of solvent gives an absorbance of 1 with a pathlength of 1 cm.) The mixtures were precipitated with ethanol, resuspended in water at approximately 100 A_{260} units/ml and dialyzed against 0.12 M phosphate buffer (pH 6.8). For each assay point, 2.5 A_{260} units were denatured at 100° for 5 min and immediately incubated at 60° to desired C_0t values. The reassociated and nonreassociated molecules were monitored radiometrically and optically after separation via 1-ml bed volume hydroxylapatite columns at 60°. In each instance, the percent reassociation was based on the total of recovered material. As controls, each set of assays included parallel reassociations of DNA isolated from a 46,XY male, a 46,XX female, and *Escherichia coli*. The final C_0t value (4×10^3 to 10^4) obtained with each DNA tested exceeded that necessary to reassociate half the single-copy sequences present. At this C_0t value, the maximum self-reassociation of ^{3}H-it-Y sequences was about 8%.

Other Assays. H-Y antigen was determined (5) and chromosomes were stained for 5-methylcytosine (4) as previously described.

RESULTS

Results of it-Y [^{3}H]DNA reassociation assays with excess whole genome DNA from variant and normal individuals are seen in Fig. 1 and numerically summarized along with other features in Table 1. Reassociation with 46,XY DNA is plotted as the mean of multiple assays in Fig. 1. In Fig. 1*A* the relative reassociation rate of it-Y [^{3}H]DNA with DNA from variant individuals was standardized by comparison with parallel reassociation assays with 46,XY DNA. The reassociation assays with DNA from different variant individuals were compared by plotting their reassociation rates relative to the reassociation curves derived from the mean reassociation values of it-Y [^{3}H]DNA with DNA from the normal males. Data in Fig. 1*B* and *C* were not so normalized. In all instances, maximum reassociation was set to 100% and percent reassociation was expressed as a fraction of the maximum reassociation obtained with 46,XY DNA in each set of assays (Fig. 1 legend).

Based on $C_0t_{1/2}$ values there is—relative to 46,XY DNA—an approximately 1.9-fold acceleration in reassociation rate of it-Y [^{3}H]DNA to DNA from individuals 1 and 2 who bear two long arms of the Y chromosome (Fig. 1*A*). This near doubling of reassociation rates with DNA from the 47,XYY male (individual 2) and from the 46,X,i(Yq) female (individual 1) indicates that the number of it-Y sequences is approximately doubled in both genomes. The reassociation rate of ^{3}H-it-Y sequences with DNA from each of two females who have translocations of Yq to other chromosomes (individuals 5 and 6) was identical to that seen with DNA from normal 46,XY men (Fig. 1*B*).

Reassociation of it-Y [^{3}H]DNA with DNA from each of two 46,XYq− males (individuals 3 and 4) who lack approximately half of the Y chromosome long arm indicates there has been an overall loss from these genomes of more than half of all it-Y sequences (Fig. 1*B*). The decreased rate of reassociation ($C_0t_{1/2}$ analysis, Table 1) suggests that the reiteration frequencies of the it-Y sequences present in these genomes is about half that seen in a normal 46,XY male. The final reassociation value obtained—90%—suggests that particular families of it-Y sequences, about 10% of the total, are completely lacking in these genomes. Thus, approximately half of all it-Y sequences are present in the deleted fragment.

DNA isolated from individuals 7–10 reassociated it-Y [^{3}H]DNA no better than did DNA from either normal 46,XX women or *E. coli* (Fig. 1*C*). Accordingly most, if not all, it-Y DNA sequences are absent from these genomes.

DISCUSSION

Reassociation assays (Fig. 1*A* and *B*) of it-Y [^{3}H]DNA with DNA from the 46,X,i(Yq) female (individual 1), the 46,X,t(Y;

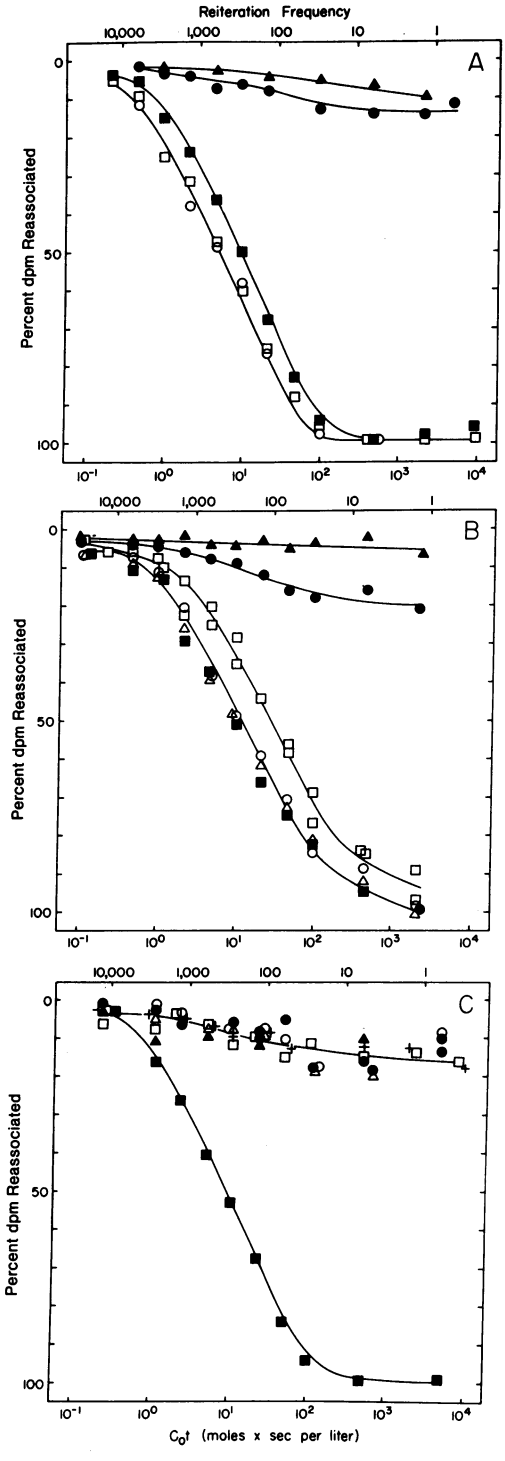

FIG. 1. Reassociation assay between trace levels of it-Y [^{3}H]DNA and excess leukocyte DNA from each of the following individuals. *A*. 46,XY (■); 46,XX (●); *E. coli* (▲); 47,XYY (individual 2) (□); 46,X,i(Yq) (individual 1) (○). *B*. 46,XY (■); 46,XX (●); *E. coli* (▲); 46,XYq− (individuals 3 and 4) (□); 46,X,t(X;Y) (individual 6) (○); 46,X,t(Y;17) (individual 5) (△). *C*. 46,XY (■); 46,XX (●); *E. coli* (▲); 45,X/45,X,t(Y;22) (individual 8) (□); 46,XX,15p$^+$,22p$^+$ (individual 7) (○); 46,XX male (individual 9) (△); 45,X/46,X,?Y (individual 10) (+). Reassociation with DNA from variant individuals was assayed in parallel with 46,XY DNA. In each instance 100% reassociation was defined as the maximum reassociation to 46,XY DNA. The range of maximum reassociation of it-Y [^{3}H]DNA for each curve was: 46,XY = 51.5–64.0%; 46,XX = 5.0–13.5%; 47,XYY = 47.5–64.5%; 46,XYq− = 43.1–60.8%; 46,X,i(Yq) = 54.5%; 46,X,t(Y;17) = 62.7%; 46,X,t(X;Y) = 61.6%; 45,X/45,X,t(Y;22) = 9.5%; 46,XX,15p$^+$,22p$^+$ = 9.3%; 46,XX male = 14.6%; 45,X/46,X,?Y = 10.9%; *E. coli* = 6.5–8.0%. Each point of the curves plotted for 46,XY DNA in *B* and *C* is the mean of at least two determinations. In *A* the 46,XY DNA curve is the mean of at least five sets of determinations. The 46,X,i(Yq) and 47,XYY curves have been normalized to this mean of five determinations. The 47,XYY is the average of two sets of determinations on the same individual and the 46,XYq− is the average of separate determinations for individuals 3 and 4. Each point of the curves plotted for 46,XX and *E. coli* DNAs in *A* and *C* is the mean of at least two determinations. Each of the other plots is derived from a single set of determinations. In all instances, 80–85% of the unlabeled DNA (monitored at A_{260}) had reassociated at C_0t 10,000. Reiteration frequencies given at the top of the figure were calculated on the assumption that the $C_0t_{1/2}$ of single-copy sequencies from man relative to *E. coli* is 3×10^3.

17) female (individual 5), and the 46,X,t(X;Y) female (individual 6), together with correlations (Table 1) with other Y chromosome markers—located either just proximal to the fluorescent segment (5-methylcytosine) or near the centromere (H-Y antigen)—are consistent with localization of most it-Y DNA to the long arm of the Y chromosome. The corollary is that it-Y DNA is largely absent from the short arm and centromere regions. This conclusion is substantiated by reassociation with DNA isolated from individuals 8–10. These males are positive for H-Y antigen (Table 1) and in two instances (individuals 8 and 10) contain seeming Y chromosome fragments, yet all lack it-Y sequences in leukocyte DNA. Although there are uncertainties for any one karyotype analysis as it relates to the complete absence of short arm segments (individuals 1, 5, and 6) or the presence of an entire short arm (individuals 8–10), the aggregate of correlations (Table 1) leaves little doubt that the majority of it-Y species are absent from the short arm of the Y chromosome.

It-Y [^{3}H]DNA reassociates to 46,XY DNA with complex kinetics (Fig. 1). It represents a number of different families of reiterated sequences each of which has a reiteration frequency ranging from 300 to 600 copies (Fig. 1 legend). This finding, together with the calculated total of it-Y DNA, suggests that it-Y is a collection of 15–30 different families of reiterated sequences. Since it-Y sequences comprise only a small proportion of the estimated DNA within the Y chromosome long arm, data from Yq− variants, individuals 3 and 4 are compatible either with the clustering of it-Y DNA at one or more points in the neighborhood of the middle of the long arm or with a wider distribution along its length. Whatever the topography, the decreased rate of reassociation of ^{3}H-it-Y with DNA isolated from 46,XYq− (individuals 3 and 4) establishes that most it-Y sequences, i.e., $\simeq$ 90% (Fig. 1*B*) lie within both deleted and nondeleted segments of the Y chromosome in these individuals. At the same time, the lack of complete reassociation in these two instances suggests that about 10% of it-Y families are confined to the distal portion of the long arm (Fig. 1*B*). Further analysis of the distribution of it-Y sequences along the long arm of the Y chromosome will be aided by the study of additional kinds of Y-variant individuals, e.g., those in whom a known 1/4 or 3/4 of the long arm has been deleted.

The it-Y DNA described here represents a heterogeneous population of reiterated DNA families that are confined to a single chromosome. Most, in fact, appear to be localized to the long arm of the Y. This it-Y DNA is thus distinct from the general class of reiterated DNA in which a single family of sequences is apparently associated with several chromosomes (8). The correlations summarized in Table 1 establish that the bulk of this particular class of Y chromosome DNA has little to do with male determination. Despite a doubling of both the usual number of Y chromosome long arms and it-Y sequences, individual 1 exhibits a normal female phenotype. Individuals 5 and

Table 1. Human Y chromosome markers and quantities of it-Y DNA in normal and variant individuals

Individual no.	Phenotype	Karyotype*	Bright Q.M. band†	5-Methyl-cytosine‡	H-Y antigen§	it-Y DNA
—	Female	46,XX	—	—	—	0¶
—	Male	46,XY	+	+	+	1
1	Female	46,X,i(Yq)	++	N.T.‖	N.T.	1.9
2	Male	47,XYY	++	N.T.	N.T.	1.9
3	Male	46,XYq—	—	+	+	0.5
4	Male	46,XYq—	—	N.T.	N.T.	0.5
5	Female	mos45,X,t(Y;17)(Yqter → Yq11::17p13 → 17qter)/46,X,t(Y;17)(Yqter → Yq11::17p13 → 17qter;17pter → 17p13::Yq11 → Ypter)	+	N.T.	N.T.	1
6	Female	46,X,t(X;Y)(:Xq22 → Xp11 or Xpter → Xq11::Yq11 → Yqter)	+	N.T.	+	1
7	Female	46,XX,15p+,22p+	—	—	—	0
8	Male (aberrant)	mos45,X/45,X,t(Y;22)(Yp11 → Ypter or Yq11 → ?::22pter → 22q13)	—	—	+	0
9	Male (Kallman's syndrome)	46,XX	—	—	+	0
10	Male (aberrant)	mos45,X/46,X,?Y	—	N.T.	+	0

* Ref. 18.
† Ref. 3, brightly quinacrine fluorescent distal segment.
‡ Ref. 4 and unpublished data of R. R. Schreck, O. J. Miller, and B. R. Erlanger.
§ Ref. 5
¶ The relative amount of it-Y DNA in each genome was estimated from the $C_0t_{1/2}$ for its reassociation of it-Y [^{3}H]DNA compared to the $C_0t_{1/2}$ for reassociation of it-Y [^{3}H]DNA with DNA from normal 46,XY men. The $C_0t_{1/2}$ values, determined as the C_0t necessary to achieve 50% of maximum reassociation (Fig. 1), were: 47,XYY and 46,X,i(Yq), $C_0t_{1/2}$ = 5.4; 46,XY, $C_0t_{1/2}$ = 10; 46,X,t(Y;17) and 46,X,t(X;Y), $C_0t_{1/2}$ = 10; and 46,XYq−, $C_0t_{1/2}$ = 20. The value 0 represents no reassociation of it-Y [^{3}H]DNA beyond that to 46,XX or *E. coli*.
‖ N.T. = not tested.

6 are also female yet have normal quantities of it-Y sequences and possess most of the long arm. Individuals 8–10 have many aspects of maleness, in particular, H-Y antigens and seeming fragments of Y chromosome (individuals 8 and 10), yet lack detectable it-Y sequences in leukocyte DNA. Given the reiteration frequency of it-Y DNA and the excess of patient-derived driver DNA used to assay their reassociation, a 100-fold reduction of all it-Y sequences would have been detectable. While we might still salvage a maleness role for it-Y sequences in any one individual by supposing that the individual is mosaic and that leukocyte DNA is unrepresentative of germinal cells, such an explanation seems implausible when our patients are considered in the aggregate. A male-determining function is thus excluded for the bulk of the it-Y sequences. It is not, however, necessarily precluded for all species of them. This possibility arises from the fact that the sensitivity of reassociation assays (Fig. 1) is such that any single family within the ^{3}H-it-Y ensemble, i.e., $\leq 1/15$ of the whole, might have been present in individuals 8–10 but remained indistinguishable from the level of background reassociation obtained with DNA from normal women.

Quite apart from applications to potential function, mutant-based tactics adopted here can also be used to purify particular species of it-Y sequences. For example, by methods analogous to those used to isolate it-Y sequences, it should be possible to isolate sequences missing from 46,XYq-genomes, e.g., the sequences in individuals 3 and 4. Additional fractionation may become possible as Y chromosome variants with different size long arms become available for study. Still other fractionation schemes may develop from the use of restriction enzymes. Indeed, Cooke has recently identified two Y-chromosome-specific fragments after cleavage with restriction enzyme *Hae* III (19). One of these is about 3400 nucleotides in length while the other is about 2500 nucleotides long. The relationship between these fragments and those described here is unclear. Cooke suggests that the larger of the *Hae* III fragments consists of simple tandemly repeating DNA which corresponds to about 50% of the Y chromosome. This interpretation is not in agreement with the kinetic complexity, the reiteration frequencies, or the distribution of the it-Y sequences reported here. For example, it is unlikely that Yq-individuals (individuals 3 and 4) would seem to lack particular species of it-Y sequences if such sequences were no more than a part of a simple tandem repeat.

Our studies thus identify a population of reiterated DNA families (it-Y DNA) that are localized to a single arm of a single chromosome. This collection of reiterated DNA families have the characteristics of neither tandemly repeated simple sequence DNAs (20) nor of the general class of reiterated DNAs wherein a single family is seemingly associated with several chromosomes (8). As described here, the conjoint use of mutants and Y-specific probes creates a model scheme for mapping the chromosomal organization of various classes of DNA.

This work was supported by National Institutes of Health Grants HL 15026 and GM 19489 to S.H.B., now an Investigator of the Howard Hughes Medical Institute; NIH Grant GM 22966 and an award from The National Foundation to O.J.M.; NIH Grant HD 00339 to W.R.B.; NIH Grants CA 08748, AI 11982, and HD 10065 to S.S.W.; and an award from the William Penn Foundation to D.S.B.

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