THE USE OF MOLECULAR HYBRIDIZATION TO EXPLORE GENETIC
RELATIONSHIPS

Progress Report

July 1, 1974 - March 31, 1975

Kimball C. Atwood, M.D.

MASTER

Department of Human Genetics and Development
College of Physicians and Surgeons
Columbia University
New York, New York

July 1, 1975 - June 30, 1976

PREPARED FOR THE U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION
UNDER CONTRACT AT(11-1)-3133

DISTRIBUTION OF THIS DOCUMENT UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

PROGRESS REPORTrDNA in Primates

The reasons for investigating the rDNA distribution in Primates have been set forth in the previous Progress Report. Since then the rDNA has been mapped in two further representatives of the Pongidae, the pygmy chimpanzee (Pan paniscus) and the mountain gorilla, with most unexpected results. Our present knowledge of this distribution in higher Primates is shown in the following table, where "+" indicates presence of rDNA in the chromosomes homologous to the human chromosomes numbered in the top row:

Human chromosome:	13	14	15	21	22	2p or 18
Man	+	+	+	+	+	-
Chimpanzee	+	+	-	+	+	+
Pygmy chimp, animal (a)	+	-	-	+	+	+
" " " (b)	+	±	-	+	+	±
Gorilla	-	-	-	+	+	-
Lar gibbon	Single site, no apparent homology to human chromosomes.					

The two pygmy chimpanzees differed from P. troglodytes in having all, or nearly all, the rDNA in only four chromosome pairs. In one animal the homologue of human chromosome 14 was lacking in rDNA, whereas the other animal appears to be heterozygous for two deficient chromosomes. This variation in rDNA distribution is much more extreme than we have observed among human subjects and in P. troglodytes. To our surprise, the mountain gorilla has rDNA on only the two smallest pairs of acrocentrics, corresponding to human chromosomes 21 and 22. The gorilla has several medium-sized

acrocentrics that show quinacrine bright fluorescence, thus excluding the previously entertained notion that such fluorescence, on chromosomes other than the Y, was correlated with the presence of rDNA.

The single-site distribution in Catarrhine monkeys has been further confirmed in the baboons Papio papio and P. hamadryas, where the rDNA is confined to the "marker" chromosome pair as in the rhesus monkey. Among new-world Primates, we have examined only a spider monkey (Atelus sp.) and a marmoset (Callithrix jacchus). Atelus has a single site as in old-world monkeys, but the marmoset has a multisite distribution; we have not yet established the exact number. Associations and connectives continued to be found in all of the Primates examined, and chromosomes without rDNA were not found in associations even though homologous to chromosomes that had rDNA in another species. We have not yet established a proportionality between the amount of rDNA in a given chromosome and the frequency of its participation in associations. It appears that a multisite distribution of rDNA must have been present in a common ancestor of the Pongidae and Hominidae, and that several gains and losses of rDNA have occurred with respect to particular chromosomes since these two primate groups became separated. The multisite distribution in the marmoset must be considered to have arisen independently, as it has in several non-Primate groups.

Human 5S RNA site

Genes for human 5S RNA were previously mapped in a subterminal position in chromosome 1 by means of hybridization in situ with ^3H or ^{125}I labelled human 5S RNA. The arms of chromosome 1 are difficult to distinguish without banding, and in our first study the labelled arm was erroneously identified as 1p. A reliable criterion of arm identification for chromosome 1 was developed by means of plates prephotographed with quinacrine fluorescent

staining and then subjected to the hybridization procedures. The correct position of the site is in 1q, as had been suggested by Steffensen et al. No sites elsewhere were detected, but we cannot exclude the possibility of secondary sites comprising very few copies of the gene.

rDNA in the laboratory mouse

Hybridization in situ of mouse ^{125}I -rRNA to mouse chromosomes bearing cytological markers had allowed the identification of chromosomes 15, 18 and 19 as carriers of rDNA in the particular strain used. The chromosome preparations were obtained from mice heterozygous for three translocations: T(14;15)6Ca; Rb(9;19)163H; and Rb(6;15)1A1d. These mice have a complex derivation from AKR/J, CBA/H-T6, and a non-inbred stock. In strain SEC/ReJ, Elsevier et al (1975) report rDNA on chromosomes 12, 16, and 18. According to these authors SEC/ReJ has prominent constrictions near the centromeres of chromosomes 12 and 16 rather than 15 and 19. Our results implicating chromosomes 15, 18 and 19 have been rechecked and confirmed. If the report of Elsevier et al is confirmed, it will indicate that translocations with breaks within or just distal to the rDNA have occurred spontaneously in the mouse. The potential usefulness of such interstrain differences will be outlined in our proposed research.

Mapping of low-multiplicity genes by hybridization in situ

Further confirmation of the feasibility of mapping low-multiplicity genes was obtained by means of grain counts over cytologically marked mouse chromosomes where nos. 7 and 11 are known to carry the β and α globin loci, respectively. The first experiments were done with mouse reticulocyte mRNA labelled with ^{125}I to a specific activity of 3×10^8 dpm/ug. T(7;14)145 had appeared to be labelled, with 0.33 grains per unit length compared to 0.07 grains per unit length for the rest of the

chromosome complement. Since chromosome 19 carries rDNA in this stock, we considered the possibility that the labelling represented ^{125}I -rRNA contaminant despite competition by a fifty-fold excess of unlabelled rRNA. To test this point the identical procedure was carried out on slides of three translocations, Rb(9;19)163H, Rb(6;15)1A1d and T(14;15)6Ca that involve chromosomes with rDNA. These translocation chromosomes were not labelled, showing that the prior result had not been influenced by rRNA contamination.

A reservation in regard to the foregoing experiment is that recognition of the translocation chromosome bearing the arm of 7 on the centromere region of 19 is somewhat subjective since it is not a banded chromosome. Another translocation stock, PMV-3, was used in which the chromosomes can be recognized even by an untrained observer. This stock is homozygous for three Robertsonian translocations, 5;15, 11;13, and 16;17. The ^{125}I -mRNA had a specific activity of 1.4×10^8 dpm/ μg and the slides were exposed for 30 days. In these slides grains were counted over five unequivocally identifiable regions, each a known percentage of the total chromosome length. The results in 27 figures selected for clarity and not for grain distribution are shown in the following table:

Region	Percent of length	Observed no. of grains	Expected no. of grains	d^2/E
5	5.8	50	58	1.1
15	4.2	46	42	0.4
11;13	9.0	137	91	23.3
16;17	7.6	82	77	0.3
Other	73.4	692	739	3.0

$$\chi^2 = 28$$

$$P = .00002$$

The labelling of 11;13 which carries the α locus is highly significant. The plausibility of the conclusion that the excess labelling of 11;13 represents the α locus can be examined from another viewpoint. The overall efficiency of grain formation, which is the product of the proportion of genes hybridized and the grain yield per disintegration, is given by $E = C/SWD$, where C is a constant (Avogadro's number/ 1440×10^6), S is the specific activity of the probe, W the mol. wt. equivalent of the region to be hybridized (140,000 per gene in this case), and D the days of exposure per grain observed. From the table, 51 of the 137 grains in 11;13 can be attributed to the α locus on 108 chromatids, i.e., 0.47 grains per locus in 30 days, or 64 days per grain. From the equation, an efficiency of about 8% would be required if there were four structural genes at the locus. This is well within plausible limits. A plausibility estimate as in the foregoing example has been applied to each of our results on mouse and human globin loci, and all are within the plausible range. It can be concluded that mapping of low-multiplicity loci is feasible and that the assignment of human globin loci to 2q and Bq is probably correct; but however correct, the result of Price et al (1972) with a probe of very low specific activity is still not reconciled with theoretical objections.

Technical improvements in iodination of RNA

Methods of iodinating RNA have been published by several authors. We have continued to modify older techniques to increase the specific activity of the produce and allow optimal recovery of the undegraded portion of the yield. The method is adapted to carrier-free ^{125}I ; hence the concentration, 2.5×10^{-5} M, is lower than is generally used. The Na acetate buffer at pH 5 is 0.01 M, ten-fold below the concentration generally used. It was found empirically that the lower sodium concentration

favors high specific activity of product. For the same reason the concentration of TiCl_3 was raised to 0.01 M, four to five times higher than usually employed. It has been customary to stop the reaction with sulfite, but we omit this step and load the reaction mixture directly on a G25 sephadex column and recover the fast fraction. Heating to remove unstably bound iodine is done after the column step and followed by a second pronase treatment and phenol extraction. Finally the product is fractionated on a sucrose gradient. The modified procedure allows 50% recovery of RNA with 3×10^8 dpm/ug.

Formation of RNA aggregates

The aggregation of RNA into large complexes is potentially useful for in situ mapping of low-multiplicity genes, or as a "handle" for the isolation of genes that are still attached to adjacent regions such as "spacer" sequences, medium repetitive sequences, or neighboring structural genes. Such secondary isolates would provide much-larger probes for hybridization in situ than most of the sequences (e.g., mRNA) that can be primarily isolated. Of a number of possibilities tried, one has given reproducible results. Cross-linked aggregates of RNA greater than 70S have been made from 18S rRNA by a reaction with the bifunctional mustard Bis-(2-chloroethyl)methylamine HCl (HN2). Such aggregates comprise more than ten molecules of the original RNA. The conditions under which the reaction gives reproducible yields are 400 ug RNA/ml in 51% methanol at pH 7.5 with 10 mg/ml HN2, 0.1 ml total volume. After 30 min. at 55°C the mixture is chilled, diluted 4:1, and placed on a sucrose gradient. HN2 attaches to the N7 position of guanine and thus should not seriously interfere with hybridization.

Genetics of rDNA in Drosophila

The bobbed locus in *Drosophila* provides a model system for understanding the mechanisms whereby optimal multiplicity of rDNA is maintained. The previous Progress Report described experiments on magnification of bobbed in ring chromosomes. A new result of these experiments concerns the effect of magnifying conditions on the sex ratio in the progeny of males with ring X chromosomes; it can be explained in the context of the previous studies.

Most bobbed alleles are partial deficiencies of rDNA. Magnification is a reversion of bobbed to wild type initiated with very high frequency in males that have a strongly bobbed phenotype. The phenotypic reversion is accompanied by an increase in rDNA. Unequal exchange has long been regarded as a possible source of differences in the length of the tandem rDNA segment. Tartof (1974) concluded that unequal sister chromatid exchange is the mechanism of magnification. That conclusion rested largely on the inference that reduced bobbed alleles (i.e. more severe than the parental allele and having less rDNA), which appear during magnification, are reciprocal products of the same events that generate magnified bobbed. Tartof's experiments included one in which a bobbed allele in a ring X did not magnify. The purpose of using ring chromosomes is to observe the effect of magnifying conditions where the products of odd-numbered sister chromatid exchange would be lost as compound dicentric rings. Our previous results are summarized as follows:

1. Magnification is completely inhibited in ring X or Y chromosomes.
2. The same alleles that do not magnify in rings show normal magnification when the rings are spontaneously or intentionally opened.

3. Reduced bobbed alleles are found in the progeny of ring X males under magnifying conditions (presence of Ybb) where magnified bobbed is totally absent. Under nonmagnifying conditions (presence of Ybb+) no reduced bobbed alleles appear.
4. Incorporated tritium partially relieves the inhibition imposed by closure of the chromosome, allowing recovery of magnified bobbed in ring chromosomes.
5. Ring chromosomes obtained from stocks kept as rings for many years carried bobbed or bobbed-lethal alleles although the rings originally had wild-type alleles.

These results allow a strong inference that sister chromatid exchange is required for magnification. The tritium effect is attributed to induced exchanges that convert compound dicentric rings into separable rings. In view of the complete absence of magnification in rings without tritium, the exchanges enhanced by magnifying conditions must be confined to the rDNA segment; otherwise some of the exchanges within rDNA would be accompanied by an exchange elsewhere which would, with an expectation of $\frac{1}{2}$, give separable products. Reduced bobbed alleles in rings are attributed to exchanges that are nonreciprocal with respect to the sister chromatids and result in loss of rDNA as small acentric rings. The reduced products of reciprocal exchanges are not recovered in rings. In open chromosomes, the small proportion of reduced alleles recovered in comparison to the proportion magnified is consistent with premeiotic events; many of the reduced alleles would be eliminated by selection in premeiotic cell lines.

We have found that magnifying conditions shift the sex ratio in the progeny of ring X males in the direction of ring loss, whereas the same

conditions have no effect on the sex ratio in the progeny of males with open X chromosomes. This is a manifestation of the same effect that prevents magnification in rings. It indicates that some of the events are meiotic, since the nullo-X cells resulting from ring loss would not survive through premeiotic cell lines. The sex ratio shift is a sensitive index of magnifying conditions; both equal and unequal exchanges contribute to it. On the assumption that two divisions are involved, a frequency of about 8% per division of odd-numbered exchanges in rDNA is required to account for the change in sex ratio.

We surmise that the slow changes in bobbed, which optimize the rDNA content under nonmagnifying conditions, are brought about by the same exchange mechanism as the rapid changes during magnification, but at a very much lower frequency. Thus the slow loss of rDNA from ring chromosomes that originated with a normal amount is explicable on the basis that losses are possible in rings by nonreciprocal exchange, whereas increases would always be eliminated as compound dicentric rings. The most interesting feature of this system is the complete specificity of the enhanced exchanges for the rDNA segment. It implies that a special "recombinase" is induced under magnifying conditions.

Genetics of 5S RNA in Drosophila

One of the goals of our last Research Proposal was to synthesize a better tester stock for partial 5S deficiencies than the Y-2 translocation deficiency aneuploid then in use. This has been accomplished by the use of a tandem duplication of Nix (1973) that includes wild-type alleles of Minute-173, selected as an anti-Minute. The duplication is just distal to the 5S locus in 2R. By screening for elimination of the anti-Minute

by X rays we recovered a deficiency for one of the duplicated segments that also deleted the adjacent 5S locus. When this deletion was in combination with a partial 5S deficiency the phenotype, resembling a severe bobbed, was such that magnification should have occurred. None, however, was observed. This suggests that the mechanisms controlling the multiplicity of 5S DNA may differ in some detail from those acting on rDNA.