THE ISOLATION OF COMPLEMENTARY STRANDS FROM A MOUSE DNA FRACTION*

By W. G. Flamm,† Mora McCallum, and P. M. B. Walker

CHEMICAL BIOLOGY UNIT, DEPARTMENT OF ZOOLOGY, UNIVERSITY OF EDINBURGH, EDINBURGH, SCOTLAND

Communicated by James Bonner, April 3, 1967

The nuclear DNA from all mouse strains[1-6] and mouse tissue[1, 2] thus far investigated exhibits two DNA bands when centrifuged to equilibrium in a CsCl density gradient. The principal band, which comprises 90 per cent of the nuclear DNA, is found at a buoyant density 0.01 gm cm^{-3} higher than the minor component, or satellite band, as it is generally called.

Not only is this satellite DNA remarkably homogeneous in GC content,[6, 7] but it also undergoes rapid renaturation at low DNA concentrations.[6-9] The latter property has also been used to separate renatured satellite DNA from the bulk of the nuclear DNA which remains denatured after heating.[8-10] A comparison of the rates of renaturation of satellite with bacteriophage, viral, and bacterial DNA's strongly suggests that there is within the satellite part of the mouse genome a nucleotide sequence which is repeated perhaps one million times.[9]

The use of fixed-angle instead of swinging-bucket rotors has allowed this satellite band to be isolated in a native and highly purified state.[11] Further, its extreme homogeneity both in base sequence and composition suggested to us that it might be possible to isolate the complementary strands from alkaline CsCl gradients,[12] a view confirmed, during the course of this study, by Dr. H. E. Bond of the National Cancer Institute, who also had found a bimodal distribution for this fraction during analytical centrifugation in alkali.[13] We describe in this paper the method of separation and the properties of the isolated strands.

Materials and Methods.—Preparation of DNA fractions: DNA of moderately high molecular weight was extracted from mouse liver and mouse L cells by a modification of the Marmur procedure.[11, 14] Labeled DNA was obtained from mouse L cells which were grown for two to three generations in the presence of P^{32}-sodium phosphate. The mouse satellite fraction was isolated from CsCl density gradients following 60 hr centrifugation in fixed-angle rotors[11] (see legend, Fig. 1). One-tenth-ml fractions were collected from these gradients by piercing the centrifuge tube and displacing its contents with liquid paraffin, and the UV absorbance of each fraction was measured with a Beckman DB-G spectrophotometer.[11] Pooled satellite fractions were then diluted to a density of 1.2 gm cm^{-3} with a suitable buffer solution and the DNA was pelleted by an 18-hr centrifugation at 50,000 rpm. In this way small quantities of DNA may be concentrated and the desired buffer system obtained. When very low salt concentrations were required, the process was repeated to further eliminate the presence of Cs ion. As has been shown,[7] this procedure is effective and does not change the weight average molecular weight of our DNA fractions.

Alkaline CsCl gradients for the fixed-angle rotor were prepared by adding 100 μl of 1 *N* NaOH to 3.3 ml of solution containing 18–100 μg of satellite DNA and 500 μg of sodium lauryl sulfate (SLS) in 0.01 *M* Tris-HCl, pH 8.5. CsCl was then added to establish an initial density of 1.760 gm cm^{-3}. The final pH ranged from 12.4 to 12.7. The presence of detergent (SLS) is critical in polypropylene tubes since a selective adsorption of the heavy strand together with a lower resolution is otherwise observed. Polypropylene tubes are used since at high pH, absorbing material elutes from nitrocellulose. Similar gradients were prepared for model E analysis and the UV exposures were analyzed with a Joyce-Loebl microdensitometer.

Melting temperature: Thermal denaturation of DNA was followed in a Beckman DB-G spectro-

photometer using a modified cell holder and slit-width device developed in this laboratory, in which small sample volumes (0.4 ml) in ordinary quartz cells can be analyzed. The samples were overlaid with liquid paraffin and stoppered to prevent evaporation. The temperature rise was from 0.25 to 0.5°C per minute, recorded directly from the sample cell.

Base ratios: Nucleotide compositions were determined following enzymatic breakdown of P^{32}-labeled DNA to 5-monophosphates. Individual nucleotides were isolated by chromatography on Dowex 1-X8 formate columns as described elsewhere[15] and their molar ratios assessed from radioactivity measurements.

Chromatography on hydroxyapatite columns: Solutions containing 0.04 μg DNA/ml in 0.12 *M* sodium phosphate buffer were heated for 10 min at 100°C, then immediately transferred to a 60° water bath and incubated for the periods indicated in Figures 5 and 6. Following these incubations, reaction mixtures were layered onto hydroxyapatite columns and fractionated by stepwise elution with 0.12 *M*, 0.16 *M*, and 0.3 *M* sodium phosphate buffer, pH 6.8, as has been described.[16] Column temperatures were maintained at 70°C.

Results.—Separation of mouse satellite DNA from the principal DNA band is readily achieved by CsCl equilibrium centrifugation in fixed-angle rotors (Fig. 1*A*), in which an enriched satellite fraction (shaded area of Fig. 1*A*) is further purified by a second centrifugation (Fig. 1*B*). Analytical centrifugation of the fractions

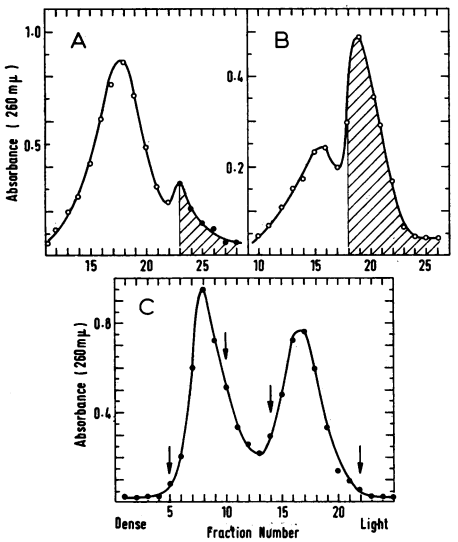

FIG. 1.—Banding pattern of mouse DNA in CsCl density gradients; (*A*) whole mouse DNA and (*B*) those fractions shaded in *A* recycled. Centrifugations in both *A* and *B* were conducted at 25°C in an M.S.E. 50 rotor (fixed-angle) operated at 35,000 rpm for 60 hr. The fractionation pattern of satellite DNA (from the shaded area, *B*) in an alkaline gradient after centrifugation in the same rotor for 24 hr at 42,000 rpm is illustrated in *C*. The fractions shown between each set of arrows were pooled for experiments on the isolated H and L bands. Fractionation and analysis were as described in *Materials and Methods*.

shown shaded in Figure 1*B* indicates that they are free of contamination by the major band and form a single peak at a buoyant density of 1.691 gm cm^{-3} (Fig. 2*a*).

When this purified satellite DNA is recentrifuged in an alkaline CsCl gradient, two new bands at buoyant densities of 1.720 and 1.752 gm cm^{-3} (Figs. 1*C* and 2*b*) appear. Since the pH of this gradient was sufficiently high (approximately 12.5) to dissociate a DNA duplex into single strands,[12] the appearance of two bands in the alkaline gradient suggested that the ratio of purines to pyrimidines may be different in each strand,[17, 18] and this is convincingly shown in the base compositions of the two strands (Table 1). Furthermore, the L and H strands are complementary in nucleotide composition.

If the individual strands are rerun in a neutral CsCl gradient in the analytical centrifuge, they form single bands at characteristic positions (L strand = 1.699 gm cm^{-3}; H strand = 1.723 gm cm^{-3} with a shoulder at 1.717 gm cm^{-3}). When

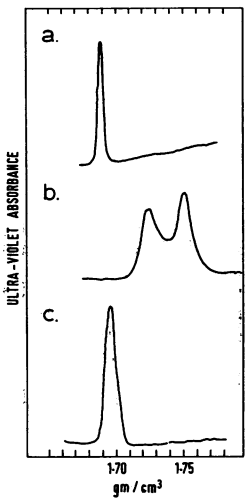

FIG. 2.—Microdensitometer traces of mouse satellite DNA centrifuged to equilibrium in (*a*) neutral, (*b*) alkaline, and (*c*) neutralized CsCl. All samples were centrifuged in the Spinco model E for 24 hr at 44,770 rpm. Initial densities at 25°C were (*a*) 1.707, (*b*) 1.750, and (*c*) 1.702 gm cm^{-3}. Buoyant densities were determined from root mean square positions and are expressed in terms of the initial density of the solution at atmospheric pressure (see Vinograd and Hearst[21]).

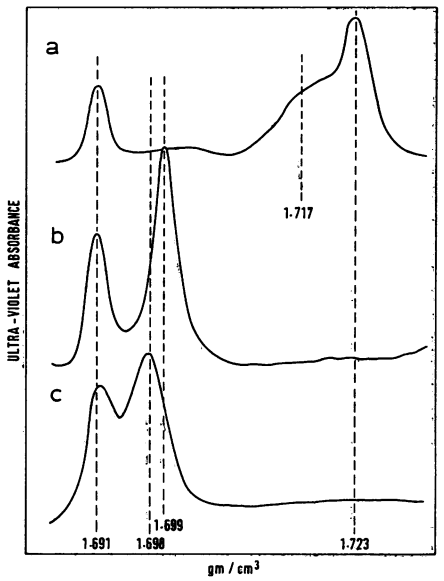

FIG. 3.—Microdensitometer tracings of (*a*) isolated H strand, (*b*) isolated L strand, and (*c*) a mixture of H and L strands incubated for 5 min at 60°C in 0.12 *M* sodium phosphate buffer and centrifuged to equilibrium in a neutral CsCl gradient. Initial densities were 1.719, 1.714, and 1.713 gm cm^{-3}, respectively. Native mouse satellite DNA (1.691 gm cm^{-3}) served as a marker. Conditions of centrifugation were as described in Fig. 2.

equimolar quantities of H and L strands are mixed and centrifuged in neutral CsCl, a single band reforms with a buoyant density (1.698 gm cm^{-3}) near that of native satellite (Figs. 2*c* and 3*c*).

The melting curves of isolated H and L strands are those characteristic of single-stranded polynucleotides (Fig. 4*A*). Furthermore, when equimolar proportions of the two strands are incubated at 60°C, they renature as shown by a melting profile (Fig. 4*B*), which is very similar to that of native satellite after it has been heated only and then allowed to renature. Both of these renatured DNA's melt within a temperature range of 10°C and possess a T_m of approximately 69°C as compared to 74.5°C for native satellite.

As has been shown by Walker and McLaren,[10] denatured DNA can be separated from either native or renatured DNA by chromatography on hydroxyapatite

TABLE 1

BASE COMPOSITION OF MOUSE SATELLITE

Bases	Mole %: Duplex	H strand	L strand
G	16.4 ± 0.2	12.5 ± 0.3	19.9 ± 0.0
A	32.2 ± 0.3	20.7 ± 0.2	44.2 ± 0.3
C	17.8 ± 0.4	22.1 ± 0.4	14.3 ± 0.3
T	33.6 ± 0.2	44.7 ± 0.4	21.6 ± 0.0
Purine/pyrim.	0.95	0.50	1.79
G + C (%)	34.2	34.6	34.2

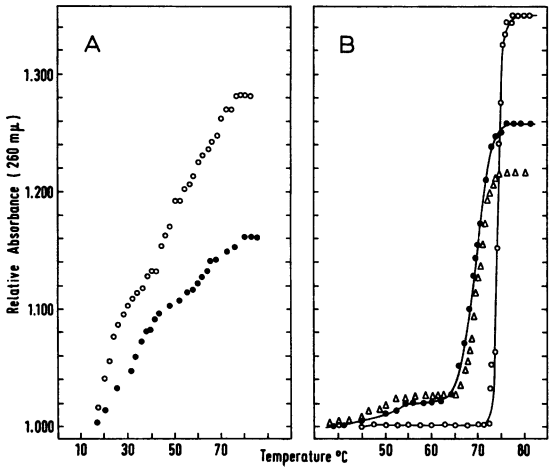

FIG. 4.—Melting curves of satellite DNA in 0.06 *M* sodium phosphate, pH 6.8: (*A*) isolated L strand (*open circles*) and isolated H strand (*closed circles*); (*B*) native satellite (*open circles*), heat-denatured satellite incubated for 5 min at 60°C (*closed circles*), and a mixture of L and H strands similarly incubated (*triangles*).

columns. Depending upon their degree of secondary structure, DNA molecules will either elute from the column at low salt concentrations (denatured molecules) or at high salt concentrations (native or renatured DNA). When the columns are operated at high temperatures, they provide an excellent means of detecting the presence of DNA duplexes.[16] By applying this procedure to the individual L and H strands, we find that approximately 80 per cent of either strand remains in the denatured fraction after a 4.5-hour incubation (Fig. 5*A*). As expected, the mixture of L and H strands, like heat-denatured satellite DNA, elutes at high salt concentration following a 4.5-hour incubation at 60°C (Fig. 5*B*).

These results are further reinforced by the kinetic experiments illustrated in

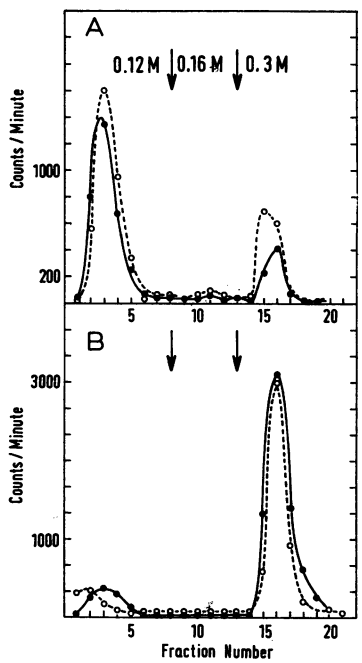

FIG. 5.—Stepwise elution of DNA from hydroxyapatite columns after a 4.5-hr incubation of 0.04 μg DNA/ml in 0.12 *M* sodium phosphate buffer. Incubation temperature was 60°C whereas column temperatures were maintained at 70°C. (*A*) Isolated H strand (*open circles*) and isolated L strand (*closed circles*); (*B*) mixture of H and L strands (*closed circles*) and heat-denatured satellite (*open circles*).

Figure 6, in which we exploit the ability of hydroxyapatite to separate denatured and renatured fractions of isotopically labeled DNA at concentrations so low that satellite renaturation can be followed. We believe that the higher molecular weight of the L and H strands accounts for the more rapid renaturation of the mixture compared to the denatured control. A small "renaturation" of the isolated strands has been shown both in Figures 5 and 6, but its significance is not yet clear although analysis of its base composition shows that it cannot be an L + H duplex. Evidence that a high degree of specificity is involved in the renaturation process, measured by hydroxyapatite fractionation, is provided from experiments in which denatured rat DNA (100-fold excess) failed to influence the renaturation of the individual satellite strands.

Discussion.—Although individual DNA strands have been isolated from certain bacteriophages by MAK column chromatography,[17, 18] this report represents the first occasion in which single DNA strands have been obtained from a higher organism. Since it is probably true that only "simple or homogeneous DNA's "will lend themselves to strand separation, the study reported here strongly supports an earlier contention[9, 19] that mouse satellite DNA contains a very limited amount of genetic information. Further, it is now certain that satellite's ability to renature rapidly represents a highly discriminatory bimolecular reaction involving the complementary sequences of its L and H strands. In view of the fact that satellite DNA is of high molecular weight and comprises about one tenth of the total mass of the mouse genome, its apparent simplicity is a puzzling feature.

The difficult question now arises whether satellite DNA might not be a virus or provirus. Its great mass within the genome; its presence in "germ-free" mice,[9] in males and females,[9] and in all tissue and strains investigated; as well as its association with metaphase chromosomes[20] and its metabolic stability[6, 7] argue against the viral origin of satellite. Furthermore, the kinetics of its renaturation are such as to suggest that satellite consists of a single nucleotide sequence of from 300 to 400 base pairs repeated approximately a million times within the mouse genome.[8, 9] Since satellite DNA has been isolated[7] with a molecular weight of approximately 40×10^6, at least 200 repeating sequences must be strung end-to-end within such a molecule. This arrangement seems rather unlikely for viral DNA.

It should be pointed out, however, that neither rat nor guinea pig DNA forms duplexes with the nucleotide sequences of mouse satellite although they both contain rapidly renaturing fractions of some kind. Clearly, therefore, the DNA homologies which exist between these three species[19] involve sequences other than those in mouse satellite. Interesting and as yet unanswered questions concern the history of satellite's evolution and the mechanism involved in the maintainance of so many

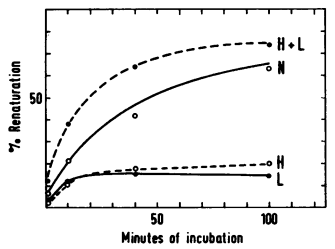

FIG. 6.—Kinetics of renaturation of L strand (*L*), H strand (*H*), a mixture of L and H strands (*H* + *L*), and heat-denatured satellite DNA (*N*). Per cent renaturation was determined from the fraction of radioactivity eluting with 0.3 *M* sodium phosphate. Conditions of the incubation and fractionation are described in Fig. 5.

apparently identical sequences within the same genome. It may be a virus and the mouse a tolerant animal, but mouse DNA presumably does more than just code for protein synthesis.

Summary.—Native satellite DNA was isolated from either mouse L cells or mouse liver by CsCl density gradient centrifugation in fixed-angle rotors. Upon recentrifugation in alkaline CsCl gradients, the satellite DNA forms two bands which are designated H and L strands. Isolated H and L strands give melting profiles characteristic of single-stranded polynucleotides and are complementary to each other in base composition. Their banding positions in neutralized CsCl density gradients and behavior on hydroxyapatite columns are consistent with our view that they represent the individual DNA strands of the satellite duplex. After mixing equimolar quantities of H and L strands, double-stranded structures rapidly reform as is shown by density gradient centrifugation, melting profiles, and behavior on hydroxyapatite columns.

We are indebted to Dr. Max Birnstiel for model E analyses.

* This work was supported by the Nuffield Foundation and the Medical Research Council.

† On leave from the National Cancer Institute, Bethesda, Maryland.

[1] Kit, S., *J. Mol. Biol.*, **3**, 711 (1961).

[2] Kit, S., *Nature*, **193**, 274 (1962).

[3] Chun, E. H. L., and J. W. Littlefield, *J. Mol. Biol.*, **7**, 245 (1963).

[4] Corneo, G., C. Moore, D. R. Sanadi, L. I. Grossman, and J. Marmur, *Science*, **151**, 687 (1966).

[5] Borst, P., and G. J. C. M. Ruttenberg, *Biochim. Biophys. Acta*, **114**, 645 (1966).

[6] Flamm, W. G., H. E. Bond, H. E. Burr, and S. B. Bond, *Biochim. Biophys. Acta*, **123**, 652 (1966).

[7] Bond, H. E., W. G. Flamm, H. E. Burr, and S. B. Bond, *J. Mol. Biol.* (submitted).

[8] Britten, R. J., in *Year Book 1964* (Washington: Carnegie Institution, 1965), p. 313.

[9] Waring, M., and R. J. Britten, *Science*, **154**, 794 (1966).

[10] Walker, P. M. B., and A. McLaren, *Nature*, **208**, 1175 (1965).

[11] Flamm, W. G., H. E. Bond, and H. E. Burr, *Biochim. Biophys. Acta*, **129**, 310 (1966).

[12] Vinograd, J., J. Morris, N. Davidson, and W. F. Dove, Jr., these PROCEEDINGS, **49**, 12 (1963).

[13] Bond, H. E., personal communication.

[14] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).

[15] Miyazawa, Y., and C. A. Thomas, *J. Mol. Biol.*, **11**, 223 (1965).

[16] Walker, P. M. B., and M. McCallum, *Biochem. J.* (in press).

[17] Marmur, J., C. M. Greenspan, E. Palacek, F. Kahan, M. Levine, and M. Mandel, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 191.

[18] Saunders, G. F., and L. L. Campbell, *Biochemistry*, **4**, 2836 (1965).

[19] McLaren, A., and P. M. B. Walker, *Nature*, **211**, 486 (1966).

[20] Maio, J., and C. Schildraut, *J. Mol. Biol.*, **24**, 29 (1967).

[21] Vinograd, J., and J. E. Hearst, *Fortschr. Chem. Org. Naturstoffe*, **10**, 395 (1962).