Self-Renaturing Fractions in the Separated Strands of Mouse Satellite Deoxyribonucleic Acid

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Pyrimidine- and purine-rich strands of *Mus musculus* satellite DNA prepared by alkaline CsCl-gradient centrifugation can self-renature to a variable extent to give partial duplexes with high thermal stability. These duplexes were purified by treatment with nuclease S_1 followed by hydroxylapatite chromatography, and have been shown by pyrimidine-tract analysis to be very similar in sequence to total reassociated satellite DNA. It is believed that the self-renaturing fractions result from variable contamination of each strand with fragments of the other, rather than from molecular inversions. The predominantly single-stranded properties of these fractions may be partly due to the ability of mouse satellite DNA strands to reassociate in non-stoichiometric proportions.

Flamm *et al.* (1967) showed that when the complementary single strands of the *Mus musculus* light satellite DNA were separated by centrifugation in alkaline CsCl density gradients, each strand contained 10–15% of a fraction able to self-renature to give structures with some of the characteristics of reassociated duplex. In a later paper (Flamm *et al.*, 1969b) these workers characterized the self-renaturing fractions in more detail, and concluded from several lines of evidence that they resulted from a small proportion of special sequences, not homologous with the major repeating sequence of mouse satellite DNA, which were covalently joined into the major sequence but periodically reversed between the H and L strands†.

These results seemed to show that the mouse satellite DNA must have a more complex structure than had hitherto been supposed (Flamm *et al.*, 1969c). Recent work on the structure and nucleotide sequence of this DNA, however, has strongly reinforced the interpretation put forward by Waring & Britten (1966), that it is composed of a tandemly repeating simple sequence (Southern, 1970; Sutton & McCallum, 1971; Pyeritz *et al.*, 1971). In the light of these developments, it was decided that the evidence for interspersed 'special sequences' needed further examination.

Materials and Methods

Preparation of ^{32}P -labelled and unlabelled mouse DNA, purification of the light satellite DNA, separa-

tion of the H and L strands and hydroxylapatite fractionations at 60°C were carried out as described by Flamm *et al.* (1969b). H strands enriched with the self-interacting fraction were prepared from the light side of the H peak in alkaline CsCl gradients, and H strands depleted of the self-interacting fraction were prepared from the heavy side of the H peak.

Analytical CsCl-density-gradient centrifugation was performed in an MSE analytical ultracentrifuge by the procedures described by Flamm *et al.* (1969a). Band positions were measured from densitometer traces of the photographs. DNA-reassociation experiments were done at 60°C in 0.12 M-sodium phosphate buffer, pH 6.8, after denaturation of the DNA at 100°C for 10 min. Crude nuclease S_1 was prepared and used as described by Sutton (1971). Nuclease-resistant 'cores' were purified by adsorption on hydroxylapatite in 0.06 M-sodium phosphate buffer at room temperature, followed by elution with 0.3 M-sodium phosphate buffer. DNA was degraded to pyrimidine tracts by treatment with 2% (w/v) diphenylamine in 66% (v/v) formic acid for 18 h at 30°C as described by Burton & Petersen (1960). Pyrimidine tracts were separated into isoplith groups by column chromatography on DEAE-cellulose as described by Petersen & Reeves (1966).

Results and Discussion

The experiments of Flamm *et al.* (1969b) established that the self-renatured fractions were partial duplexes, with chemical and physical properties (base composition, buoyant density in neutral CsCl, and hypochromicity) closer to those of the single strands from which they were derived than to those of reassociated HL duplexes. To isolate the duplex regions, we treated the self-renatured fractions with a crude preparation

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† Abbreviations: H strand, pyrimidine-rich strand; L strand, purine-rich strand.

of nuclease S_1 under conditions that result in nearly complete removal of single-stranded loops and tails (Sutton, 1971). Most of each fraction was degraded by the nuclease (Fig. 1), providing further evidence for a mainly single-stranded structure. The residues resistant to nuclease S_1 were shown to be duplex by hydroxylapatite chromatography at room temperature (Bernardi, 1965). Zone sedimentation through sucrose density gradients showed that these 'core' duplexes sedimented less rapidly through neutral sucrose than did a similar 'core' fraction from re-associated HL duplexes of satellite DNA, but their sedimentation coefficients in alkaline sucrose were both approx. 7–9 S. The 'core' duplexes were further characterized by degrading them with diphenylamine in formic acid and separating the resultant pyrimidine tracts into isoplith groups by ion-exchange chromatography. The result (Fig. 2) was clear-cut: 'core' duplexes prepared from either strand showed a distribution of pyrimidine tracts similar to that of HL duplexes. This result should be compared with experiments with total H strand and total L strand (unpublished work by A. R. Carr-Brown, E. M. Southern & P. M. B. Walker, cited by Walker, 1971), which have shown that most of the pyrimidine trinucleotides in mouse satellite DNA come from the L strand, whereas almost all the pyrimidine tetranucleotides and hexanucleotides come from the H strand.

These results prompted us to re-examine an experi-

Fig. 1. Action of nuclease S_1 on fractions of mouse satellite DNA

The ^{32}P -labelled satellite DNA was digested with 100 units of nuclease S_1 /ml at 37°C in 0.01 M-NaCl–0.03 M-sodium acetate buffer, pH 4.9, in the presence of $25\mu\text{g}$ of heterologous heat-denatured DNA/ml. ▲, Denatured H strands; △, denatured L strands; ●, self-renatured fraction of H strands; ○, self-renatured fraction of L strands; ■, reassociated HL duplexes.

ment of Flamm *et al.* (1969b), in which it was shown that non-radioactive H strands, when incubated with ^{32}P -labelled H strands from which the self-renaturing fraction had been removed, did not produce any further labelled duplexes. It was found that this result could only be repeated when the non-radioactive strands did not contain a self-renaturing fraction sufficient to be detected as a light shoulder in CsCl gradients (Fig. 3). When a non-radioactive H strand fraction enriched in self-renaturing sequences was used, a very large fraction of labelled duplexes was obtained (Fig. 3). These were shown by thermal-gradient elution from hydroxylapatite (Miyazawa & Thomas, 1965; McCallum & Walker, 1967) to have the same thermal stability as reassociated HL duplexes.

These results, together with the pyrimidine tract analyses of 'core' fractions, were taken as showing that the self-renaturing fractions from each strand, despite their mainly single-stranded structure, are held together by duplex regions similar in sequence to the bulk of the satellite DNA.

Two explanations for the origin of these duplexes were considered. The 'reversed sequences' hypothesis of Flamm *et al.* (1969c) could be retained if the sequences periodically reversed in each strand were pieces of the complementary strand, i.e. molecular inversions. Alternatively, 'self-renaturation' might simply be due to imperfect separation of the strands. In the latter case one would expect a correlation between the quality of the separation and the amount of self-renaturation. Examination of the results of this laboratory from 1968 to 1971 showed just such a correlation (Fig. 4). More decisively, when ^{32}P -labelled separated strands from which the self-renaturing fractions had been removed were used to assay fractions across an alkaline CsCl gradient of non-radioactive satellite DNA, the distribution of cross-reacting sequences was found to be symmetrical around the mean position of each non-radioactive strand (Fig. 5). Molecular inversions would be expected to give a minor peak of H sequences under the L strand, and a minor peak of L sequences under the H strand. It was concluded from this result that any inverted sequences must constitute less than 2% of each strand, and that the self-renaturing fractions are therefore an artifact resulting from imperfect strand separation.

The remaining problem is to explain why the self-renaturing fractions are mainly single-stranded. This has been exhaustively demonstrated by measurements of base composition, buoyant density, hypochromicity (Flamm *et al.*, 1969b), nuclease digestion (Fig. 1) and electron microscopy (P. O'Callaghan, unpublished work).

Two factors are probably involved. (1) During equilibrium-density-gradient centrifugation, those strand fragments banding well away from their mean

Fig. 2. Column chromatography of the pyrimidine isopliths from fractions of mouse satellite DNA

Nuclease-resistant 'cores' from the experiment described in Fig. 1 were isolated by hydroxylapatite chromatography, then they were dialysed against water and degraded with diphenylamine in formic acid. Pyrimidine isoplith groups were separated by linear NaCl gradient elution from columns (30 cm $\times$ 0.5 cm diam.) of DEAE-cellulose in 7 M-urea–0.1 M-sodium acetate buffer, pH 5.4. Each elution was completed at a high salt concentration (0.5 M-NaCl). Fractions (2–3 ml) were counted for radioactivity for 20 min in polythene vials by the Čerenkov method. (a), HL duplex 'core'; (b), H strand self-associated 'core'; (c), L strand self-associated 'core'. The first peak eluted from each column is inorganic phosphate; subsequent peaks are numbered according to chain length except for the material eluted with high salt (H.S.).

density would be expected to be smaller than those banding close to their mean density. A small fragment reacting with a larger complementary fragment will of course give a duplex structure with single-stranded

tails. (2) Despite experiments which led to the possibility of non-stoichiometric associations of low-molecular-weight satellite strands being discounted by Flamm *et al.* (1969b), it seems that a single L strand

Fig. 3. Reassociation of ³²P-labelled H strands with unlabelled self-renaturing fraction

(a) Analytical CsCl-gradient centrifugation of H-strand fractions enriched (top tracing) and depleted (lower tracing) in self-renaturing fractions. Centrifugation was for 20 h at 44 500 rev./min and 25°C. The initial CsCl density was 1.700 g/ml. (b) ³²P-labelled H strands with the self-renaturing fraction removed were reassociated at 0.05 μg/ml in the presence of unlabelled H-strand fractions enriched (●, ▲) or depleted (Δ) in self-renaturing fractions. Samples were withdrawn at intervals and the reassociated fraction was measured by hydroxylapatite chromatography at 60°C. Unlabelled strand concentrations: Δ, 3 μg/ml; ▲, 0.02 μg/ml; ●, 0.06 μg/ml.

Fig. 4. Correlation between strand separation and the amount of self-renaturing fraction

(a) Measurement of separation quality. This was defined through a function:

$$X = \frac{(2c)}{(a+b)} \frac{(d+e)}{(f)}$$

that takes account of variations in both the peak/trough ratio and the position of the cut. Shaded areas represent the fractions pooled as 'separated strands' from an idealized alkaline CsCl gradient. (b) Correlation between X and the amount of self-renaturing fraction. Each point is the average of both strands from an independent preparation of mouse satellite DNA. The line is the calculated linear regression.

can reassociate with several H strands to give predominantly single-stranded aggregates. In the experiment shown in Fig. 6, it was shown that when a

minority of L strands was added to a solution of H strands and banded in neutral CsCl without any prior exposure to conditions favouring reassociation, a

Fig. 5. Distribution of H and L sequences in an alkaline CsCl gradient

Fractions were assayed across a preparative alkaline CsCl gradient of purified, unlabelled mouse satellite DNA. ●, Distribution of DNA strands measured by their E_{260} ; ○, distribution of H-strand sequences measured by reassociating a 1/200 dilution of each fraction with $0.005\mu\text{g}$ of ^{32}P -labelled L strands/ml for 20 min, and then measuring the reassociated fraction by hydroxylapatite chromatography at 60°C ; △, distribution of L-strand sequences measured by reassociation with ^{32}P -labelled H strands.

roughly equimolar fraction of the H strands was moved to a density (1.711g/ml) midway between the H and L strands. When the same mixture was briefly exposed to optimum reassociation conditions and then banded, only a minority of the H strands remained at the H density, and three peaks of HL complexes could be discerned. These were centred at densities (1.699 , 1.705 and 1.711g/ml) compatible with duplexes containing H and L strands in ratios of slightly more than 1:1, 2:1 and 3:1 respectively. The latter two species presumably resulted from multiple nucleations occurring on the minority strands so rapidly as to compete with the 'zippering' process usually assumed to be faster than nucleation (Wetmur & Davidson, 1968).

A similar phenomenon was noticed during density-gradient centrifugation of several preparations of H strands enriched in the self-renaturing fraction, although in these cases all the components were closer in density to the H strand and they were not resolved in the densitometer tracings (Fig. 3). Non-stoichiometric associations of mouse satellite DNA strands have also been reported by Kurnit *et al.* (1972).

Further Discussion

The experiments presented here must be taken as refuting the proposals of Flamm *et al.* (1969*b,c*) about

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Fig. 6. Non-stoichiometric associations between H and L strands of satellite DNA

(a) Purified H and L strands in the ratio of 4:1 (w/w) were mixed in 0.01M-NaCl at room temperature, adjusted to a density of 1.700g/ml in neutral CsCl, and centrifuged to equilibrium as in Fig. 3(a). (b) A portion of the same strand mixture, to which CsCl was added only after reassociation of the strands for 30 min at 60°C in 0.18M-Na^+ . Densities were calculated from the density of uncomplexed H strands, taken to be 1.723g/ml (Flamm *et al.*, 1967).

the self-renaturing fractions in the strands of satellite DNA. Although their basic experimental observations have proved to be reproducible in this laboratory, we now interpret 'self-renaturation' as being due to imperfect separation of the strands. It is therefore no longer necessary to invoke a complex

molecular structure for the *Mus musculus* satellite DNA: all its known characteristics can be explained by a mutated, tandemly repeating simple sequence.

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