Oligonucleotides in Heterogeneous Nuclear RNA: Similarity of Inverted Repeats and RNA from Repetitious DNA Sites†

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ABSTRACT: A comparison has been made by oligonucleotide analysis of three fractions of HeLa cell hnRNA: (1) the "snap-back" fraction (ds-hnRNA, 5% of the total); (2) the fraction that self-anneals during prolonged incubation (25% of the total); and (3) the fraction that hybridizes most rapidly to an excess of HeLa cell DNA (rep-hnRNA, 10% of the total). T1 fingerprints of each of these hnRNA fractions were similar to one another and featured the largest T1 oligonucleotides of known sequence previously isolated from ds-hnRNA (Robertson, H. D., et al. (1977) *J. Mol. Biol. 115,* 571–590; Jelinek, W. (1977) *J. Mol. Biol. 115,* 591–602). When hybridized to DNA either in solution or immobilized on filters, the isolated ds-hnRNA and the rep-hnRNA fractions showed similar hybridization kinetics in the C_0t range of "intermediate" repetitive DNA sequences; the ds-hnRNA and the rep-hnRNA also self-annealed to equal extents in the absence of any DNA. DNA of all buoyant density classes contained the T1 oligonucleotides diagnostic of the ds-hnRNA and the rep-hnRNA. While hnRNA is rich in inverted repeated sequences, cytoplasmic mRNA contains far fewer such sequences.

The heterogeneous, nonribosomal portion of the nuclear RNA of HeLa cells, the hnRNA,[1] contains sequences scattered throughout the length of most, if not all, molecules that are transcribed from repetitious DNA (Darnell & Balint, 1970; Molloy et al., 1974). Such regions presumably arise from the so-called "intermediate repeat" DNA that is "interspersed" with nonrepetitious regions of mammalian DNA (Davidson & Britten, 1973). hnRNA also contains sequences that form intramolecular RNA:RNA hybrids (double-stranded RNA, ds-hnRNA) that comprise at least a portion of the repetitious sequences (Jelinek & Darnell, 1972; Jelinek et al., 1974). These ds-hnRNA sequences most probably are transcribed from "inverted repeat" DNA (Jelinek, 1977) of the type abcxyzc′b′a′ where a and a′, b and b′, and c and c′ represent complementary sequences and xyz, which can vary in length, represents the "turnaround" or non-base-paired region between the complementary sequences (Wilson & Thomas, 1974; Deininger & Schmid, 1976). The ds-hnRNA sequences from HeLa cells exhibit a relatively simple and diagnostic two-dimensional, T1 ribonuclease fingerprint containing six large, prominent oligonucleotides, "ds oligo's", whose sequences have been determined (Robertson et al., 1977). In addition, the inverted repeat DNA fraction has been shown to be considerably enriched in, and perhaps to contain exclusively, the oligonucleotides characteristic of the double-stranded hnRNA (Jelinek, 1977).

In the present work it is shown that the segments of hnRNA that hybridize rapidly to DNA (rep-hnRNA) and the sequences of hnRNA that "self-anneal" to produce an increased RNase resistant fraction (Federoff et al., 1977) both have a fingerprint very similar to the ds-hnRNA obtained from hnRNA that has not been annealed. Large poly(A)-containing hnRNA has a higher ds-oligo content than shorter poly(A)-containing hnRNA and mRNA has very little of the ds-oligo sequences. The interrelationship of the ds-hnRNA, rep-hnRNA, and "intermediate repeat" DNA is discussed. A possible role for the ds-hnRNA is suggested in light of recent work which indicates not only that hnRNA might be cleaved in the formation of mRNA (Darnell et al., 1976; Goldberg et al., 1977) but that reunion ("splicing") of RNA fragments may occur in the formation of eukaryotic mRNA (Berget et al., 1977; Chow et al., 1977; Klessig, 1977).

Experimental Procedures

HeLa cells were grown in suspension in Eagle's medium (Eagle, 1959) supplemented with 5% fetal calf serum. For labeling RNA with ^{32}P, cells were washed three times by centrifugation and resuspension in phosphate-free Eagle's medium and finally resuspended in phosphate-free medium containing 5% dialyzed fetal calf serum at 2–3 × 10^6 cells/mL. $H_3{}^{32}PO_4$ (carrier free) was added at 1 mCi/mL for 3–4 h, after which nuclear and cytoplasmic fractions were prepared from the washed cells. The cells were swollen in hypotonic buffer and disrupted by Dounce homogenization (Penman et al., 1963). The polyribosomal RNA was prepared from EDTA released ribonucleoprotein particles by phenol/chloroform extraction and the resulting RNA passed over a poly(U)-Sepharose column to select poly(A)-containing molecules; the poly(A)-containing RNA was considered mRNA (mRNA) (Molloy et al., 1974).

The nuclear RNA was extracted according to the procedure of Soeiro & Darnell (1969) and sedimented in a 15–30% sucrose gradient in 0.01 M Tris-HCl (pH 7.4), 0.05 M NaCl, 0.01 M EDTA, 0.2% $NaDodSO_4$. The RNA was collected and concentrated by ethanol precipitation; hnRNA represents all the labeled RNA larger than 20–30 S in sedimentation rate. Double-stranded regions were isolated from the hnRNA as

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[1] Abbreviations used: hnRNA, heterogeneous nuclear RNA; ds-hnRNA, double-stranded RNA from hnRNA; rep-hnRNA, regions of hnRNA molecules which hybridize most rapidly to homologous DNA; ds-oligo's, T1 oligonucleotides characteristic of ds-hnRNA; $NaDodSO_4$, sodium dodecyl sulfate.

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described previously (Jelinek, 1977) and in the Results section here. For RNA:DNA hybridization, the RNA was first fragmented to ~300 bases by incubation at 0 °C in 0.2 M NaOH (Jelinek et al., 1974) and hybridized to filter-bound DNA by incubation at 65 °C in 0.01 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0), 0.3 M NaCl, 0.2% $NaDodSO_4$, 0.01 M EDTA. The size of the RNA fragments was assayed by electrophoresis in polyacrylamide gels before hybridization. Alkali-denatured DNA was loaded onto Millipore filters by dissolving it in 0.1 × SSC, adding 0.1 volume of 1.0 M NaOH, and after 15 min at room temperature adding 50 volumes of 2 M NaCl and immediately filtering it through nitrocellulose filters. The filters were then washed with 6 × SSC, dried, and baked at 80 °C for 4 h. RNA hybrids were recovered either before or after nuclease digestion of the filters as described (Soeiro & Darnell, 1969). An excess of DNA to RNA was maintained in all experimentation by using 50 μg of HeLa DNA for each 2×10^7 cell equivalents of hnRNA (Pagoulatos & Darnell, 1970).

Hybridization in solution was accomplished in sealed glass capillary tubes. After the appropriate hybridization time the samples were dispelled into 1 mL of RNase digestion buffer (see details in legends to appropriate figures) and digested with RNase as described in the Results section. For fingerprint analysis of various RNA samples, the RNA was digested with T1 RNase and fingerprinted as described by Barrell (1971). Poly(A) was assayed in poly(A)-containing molecules as previously described (Molloy et al., 1974).

All RNases were purchased from Calbiochem: RNase A (Calbiochem cat. no. 55674), RNase T2 (Calbiochem cat. no. 556865), RNase T1 (Calbiochem cat. no. 556785).

Results

^{32}P-labeled hnRNA was prepared and two fractions were isolated for oligonucleotide comparison: (a) double-stranded RNA, ds-hnRNA, was derived by nuclease digestion of hnRNA that had been boiled and quickly cooled ("quenched"); and (b) the "rapidly" hybridizing hnRNA fraction (rep-hnRNA), presumably that fraction transcribed from repeated DNA (Darnell & Balint, 1970), was selected by hybridization to excess DNA immobilized on nitrocellulose filters (Pagoulatos & Darnell, 1970). During the preparation both fractions were treated extensively with a combination of ribonucleases A and T2 so that hnRNA fragments were obtained either from well matched RNA:RNA or RNA:DNA duplexes. In various experiments from 2–4% of the total hnRNA was recovered in the ds-hnRNA fraction and a somewhat larger amount was found in the rep-hnRNA fraction (5–6%). [The RNA hybridization to DNA immobilized on nitrocellulose filters was carried out after the RNA had been fragmented to oligonucleotides about 300 bases long since this treatment had previously been found necessary to allow the maximum amount of hnRNA to participate in the RNA:DNA hybridization reaction (Jelinek et al., 1974)]. Figures 1a and 1c show that the two-dimensional oligonucleotide patterns of the ds-hnRNA and rep-hnRNA were very similar. In particular, the seven largest prominent T1 products (numbered 1–7 in Figure 1d) from each of these independently derived hnRNA fractions formed a similar pattern in the two fingerprints. Six of these (no. 1–6) are the characteristic oligonucleotides from ds-hnRNA, the ds-oligo's, whose sequences have previously been determined (Robertson et al., 1977; Jelinek, 1977). In addition, most of the other prominent, smaller T1 products also formed oligonucleotide patterns that were similar in the two fingerprints (see lettered spots in Figure 1d). In some instances the RNA:DNA hybrids were not treated with ribonucleases (Figure 1b). Approximately two to three times as much labeled RNA was recovered from the hybrids not treated with nuclease but the fingerprint patterns of the larger T1 products (particularly no. 1–6, see Figure 1d) of this RNA were qualitatively similar to that of the RNA in the RNase-treated hybrid (compare Figures 1b and 1c); the oligonucleotides numbered 2 and 3 appeared somewhat more prominent when nuclease treatment was omitted.

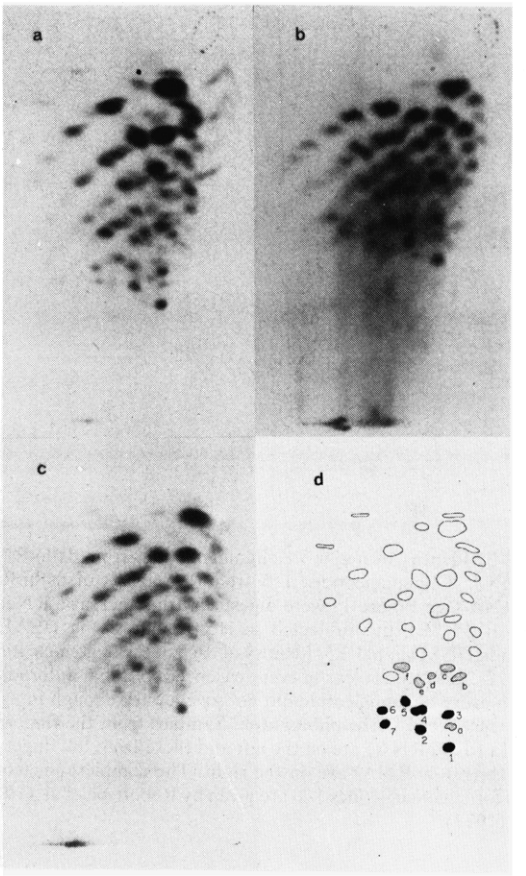

FIGURE 1: Oligonucleotide pattern of ds-hnRNA and rep-hnRNA. ^{32}P-labeled hnRNA >20S was prepared (see Experimental Procedures), divided into three parts, and treated as follows: (a) ds-hnRNA was prepared by boiling the hnRNA for 2 min in distilled water, quenching it in ice water, and digesting it with 5 μg/mL RNase A and 2.5 units/mL of T2 RNase after the addition of Tris-HCl (pH 7.4) to 0.01 M, NaCl to 0.3 M, and EDTA to 0.01 M. The RNase resistant fraction (ds-hnRNA) was selected by ethanol–cellulose chromatography (Jelinek, 1977) and subjected to T1 digestion in low salt for fingerprinting. (b) rep-hnRNA, prepared as in c but without nuclease treatment of the DNA-bearing filters; 11% of the input RNA was recovered as hybrid. (c) rep-hnRNA was prepared by alkali breakage of hnRNA to ca. 300–500 nucleotides (see Experimental Procedures) followed by hybridization to DNA immobilized on nitrocellulose filters. Ten filters, each bearing 50 μg of DNA, were hybridized to the alkali fragmented hnRNA for 20 h as described in Experimental Procedures. The filters were treated with RNase A and T2 as described in Experimental Procedures and the hybridized RNA was eluted by boiling the filters. Five percent of the input RNA was recovered as RNase resistant hybrid. This RNA and that recovered in b were digested with RNase T1 at 500 μg per mL in 0.01 M Tris-HCl (pH 7.4), 0.01 M EDTA and fingerprinted as described by Barrell (1971). Electrophoresis at pH 3.5 is from right to left and homochromatography is from bottom to top. (d) A tracing of sample a to indicate the positions of prominent T1 oligonucleotides and the numbering scheme used in the text. The fingerprinting and autoradiograph exposures for samples a and c were performed simultaneously. Sample b was prepared at the same time as a and c but was fingerprinted separately and the radioautogram was exposed ca. two to three times longer than that for a and c.

Six of the largest T1 oligonucleotides from the rep-hnRNA and the ds-hnRNA (no. 1–6) were analyzed further by treatment with ribonuclease A and electrophoresis on DEAE paper

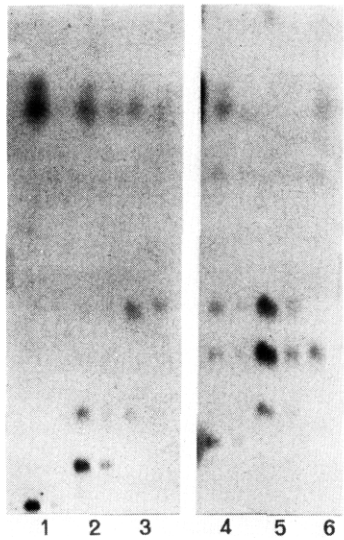

FIGURE 2: Identity of major T1 oligonucleotides from ds-hnRNA and rep-hnRNA. Oligonucleotides 1–5 from T1 digests of ds-hnRNA or rep-hnRNA (see Figure 1) were digested with pancreatic RNase (200 μg/mL at 37 °C) and subjected to electrophoresis on DEAE paper (Whatman DE 81) at pH 3.5. Identity of each of the oligonucleotide pairs (i.e, 1-1, 2-2, etc.) was clear in every case from original autoradiogram, although every oligonucleotide did not give a dark enough image to be clearly reproduced in the photograph. Samples from the fingerprint in Figure 1a (ds-hnRNA) are on the left and those from the fingerprint in Figure 1b (rep-hnRNA) are on the right. The complete nucleotide sequences for oligonucleotides 1–6 are given by Robertson et al. (1977) and Jelinek (1977).

to test whether the T1 oligonucleotides obtained from the two sources were indeed the same nucleotide sequences. The results shown in Figure 2 indicate that the correspondingly numbered T1 products from the two fingerprints yielded the same pancreatic RNase digestion products, which, upon further electrophoretic analysis after digestion by RNase T2 showed the same mononucleotide content (data not shown); the pancreatic RNase digestion products were those expected of the nucleotide sequences previously determined for these ds-oligo's (Robertson et al., 1977; Jelinek, 1977). Thus, we conclude that the corresponding largest prominent T1 products from the ds-hnRNA and the rep-hnRNA probably have the same nucleotide sequence.

Mild T1 and pancreatic RNase digestion previously were shown (Jelinek et al., 1974) to leave a residue of RNA that (a) was enriched in sequences that could hybridize rapidly to DNA and (b) had a buoyant density in Cs_2SO_4 intermediate between single- and double-stranded RNA after only 20% of the RNA was digested to acid solubility; the final 5% of completely RNase resistant material had the same buoyant density as reovirus RNA; undigested mRNA had a single-stranded density and it remained so during digestion. Thus a considerable portion of hnRNA appeared capable of participating in duplex formation. To determine the maximum amount and the oligonucleotide composition of hnRNA that could form inter- and intramolecular RNase resistant structures, self-annealing of the hnRNA and nuclease digestions were carried out under a variety of conditions. ^{32}P-labeled hnRNA was boiled for 3 min in distilled water, divided into six equal portions, and treated as indicated in Figure 3. Increasing the salt concentration in the digestion buffer from 0.3 to 0.5 M NaCl resulted in increasing the resistance to RNases A and T2 from 3.0 to 5.8%; self-annealing prior to digestion at 0.3 M and 0.5 M NaCl increased the RNase resistance to 10.1% and 27%, respectively. Two samples were digested only with RNase T1 in buffer containing 0.3 M NaCl and yielded 12% resistance without prior self-annealing and 43.7% resistance after self-annealing. The oligonucleotides in each of the six RNase-resistant fractions were further analyzed by fingerprinting. The fingerprints of the first four samples (Figures 3a, b, d, e) all yielded the characteristic ds-oligo's as the most prominent large oligonucleotides, even though the self-annealed sample digested in 0.5 M NaCl (Figure 3e) was 27% RNase resistant. In the fingerprint of samples 3c and 3f (digested with T1 RNase only in high salt prior to fingerprinting) the ds-oligo's 1–5 can easily be seen, but the T1 oligonucleotides no. 6 and 7 are not as clearly visible in the presence of the remaining large amount of heterogeneously distributed T1 RNase products.

The ds oligo's 1, 2, 3, 5, and 6 are known to be unique nucleotide sequences and the no. 4 oligonucleotide spot is a mixture of sequences, but probably not more than two (Jelinek, 1977; Robertson et al., 1977). A determination of the proportion of the total cpm found in spots 1–6, which represent about 50 nucleotides, was made by cutting them from the thin-layer plates and determining their radioactivity and the radioactivity on the remainder of the plates by scintillation counting. T1 products 1–6 accounted for 0.84% of 3b (no self-annealing, digestion in 0.5 M NaCl, 5.8% RNase resistant), 1.07% of 3d (self-annealed, digestion in 0.3 M NaCl, 10.1% RNase resistant), and 0.81% of 3e (self-annealed, digestion in 0.5 M NaCl, 27% RNase resistant). Thus the complexity of the total annealed RNase resistant sample would seem to be only about 5000 nucleotides (50 × 100), although the RNase resistant fraction made up 27% of the total hnRNA, which is estimated to have a complexity of 8×10^7 bases (hnRNA has a minimum complexity equal to ca. 10% of that of the HeLa cell genome which is $3\text{–}6 \times 10^9$ base pairs; Scherrer et al., 1970; Soeiro & Darnell, 1969, 1970).

Presence of ds Regions in Poly(A)-Containing Nuclear RNA and Cytoplasmic RNA. Approximately 20% of hnRNA molecules can be selected by virtue of a segment of poly(A) at their 3′ ends (Molloy et al., 1974). The poly(A)-terminated hnRNA molecules contain regions that hybridize "rapidly" to DNA, with a rising concentration as a function of distance from their 3′ ends. To determine whether the ds-oligo's diagnosed by fingerprint analysis followed this same general pattern of sequence distribution, ^{32}P-labeled, poly(A)-containing hnRNA molecules were prepared, denatured with 90% Me_2SO and separated into three size classes by zonal sedimentation: small (10–16 S), medium (16–25 S), and large (25 S and larger). The RNase resistance per poly(A) unit (resistance/chain) in the three size classes of hnRNA (Table I) showed that per molecule the short poly(A)-containing molecules had only ca. 7% as much RNase resistance and the medium sized molecules had ca. 25% as much RNase resistance as the large molecules. Each of the three RNase resistant fractions was subjected to T1 digestion and two-dimensional analysis (Figure 4). While all three fingerprints showed the characteristic pattern featuring the longest prominent ds-oligo's, the larger molecules contained the highest concentration of the ds-oligo's. The medium sized molecules had only 20% as much of the ds-oligo's as did the large molecules, while the small molecules contained too little radioactivity in the RNase resistant fraction for the ds-oligo's to be measured. This increasing content of RNase resistant fraction in increasingly larger poly(A)-terminated hnRNA molecules parallels the previously described results that showed the larger poly(A)-terminated hnRNA molecules to be enriched in regions of "rapidly" hybridizing RNA (Molloy et al., 1974).

Polyribosomal, poly(A)-terminated RNA, mRNA, was

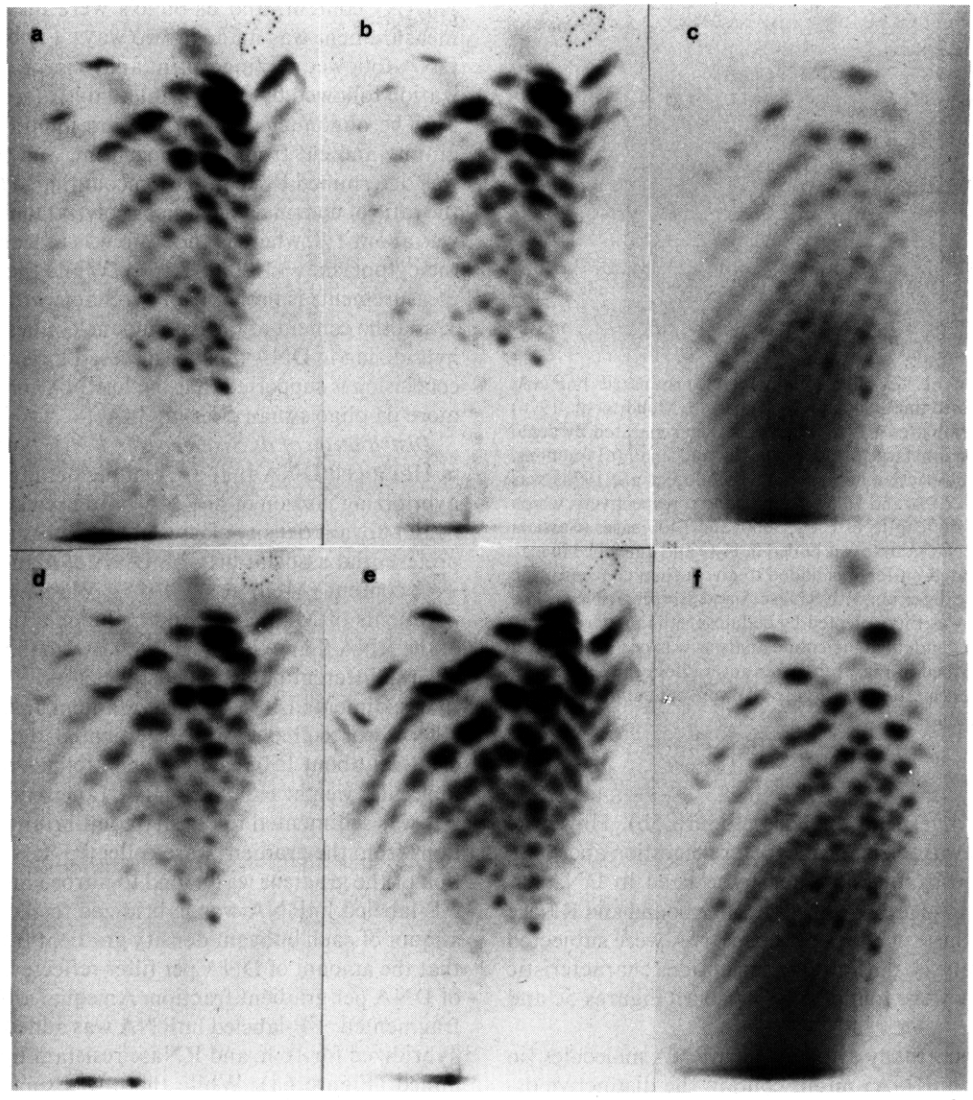

FIGURE 3: T1 fingerprints of oligonucleotides in ds-hnRNA from self-annealed hnRNA. [^{32}P]hnRNA was prepared from 4×10^8 cells and either boiled in H_2O, quenched and digested, or self-annealed overnight (RNA from 0.7×10^8 cells, in 0.05 mL, 2 × SSC, 65 °C) before digestion and oligonucleotide analysis. Percent of sample remaining acid precipitable after digestion is given for each sample: (a) boiled, digested with RNases A and T2 in 0.3 M NaCl; 3.0% RNase resistant; (b) boiled, digested with RNases A and T2 in 0.5 M NaCl; 5.8 RNase resistant; (c) boiled, digested with RNases T1 in 0.3 M NaCl; 12.0% RNase resistant; (d) self-annealed digested with RNases A and T2 in 0.3 M NaCl; 10.1% RNase resistant; (e) self-annealed digested with RNases A and T2 in 0.5 M NaCl; 27.0% RNase resistant; (f) self-annealed digested with RNase T1 in 0.5 M NaCl; 43.7% RNase resistant. The fingerprinting of samples a, b, d, and e was performed simultaneously but radioautography for a was three times longer than for b, d, and e; samples c and f were subjected to fingerprinting and radioautography separately.

TABLE I: Content of ds-hnRNA in hnRNA of Various Sizes.[a]

sample	*S* value	total cpm	cpm in poly(A)	cpm in ds-hnRNA	Relative content of ds-hnRNA (col 4/col 3)
large	25–45 (7050)	1.5×10^6	5.0×10^4	8.7×10^4	1.74
medium	16–25 (2600)	1.7×10^6	1.5×10^5	6.6×10^4	0.44
small	10–16 (1400)	1.2×10^6	1.9×10^6	2.4×10^6	0.126

[a] Poly(A)-terminated, ^{32}P-labeled hnRNA was selected by poly(U)-Sepharose chromatography, denatured in 90% Me_2SO, and sedimented to prepare three size classes. Column 1 shows *S* values measured from ribosomal markers and approximate chain lengths (in parentheses) calculated from poly(A) content (poly(A) in nuclear RNA is ca. 230 nucleotides long; Sawicki et al., 1977). Three measurements were made on the RNA from each of the three size classes. Columns 3, 4, and 5 list total cpm, cpm in poly(A), as determined by polyacrylamide gel electrophoresis of RNase A resistant RNA (Jelinek et al., 1974), and cpm of ds-hnRNA as measured after the RNA was boiled in distilled water, chilled, adjusted to 0.3 M NaCl, digested with RNases A and T2, and the RNAse resistant fraction purified by ethanol/cellulose chromatography (Jelinek, 1977). The relative content of ds-hnRNA is obtained by dividing column 5 by column 4.

prepared to determine whether any of the ds-oligo sequences could be found in these molecules. A sample of the mRNA preparation was denatured by boiling and immediately digested with T2 and pancreatic RNases. By comparison with hnRNA, virtually none (less than 0.1%) of the mRNA remained acid precipitable and no distinct, large oligonucleotides

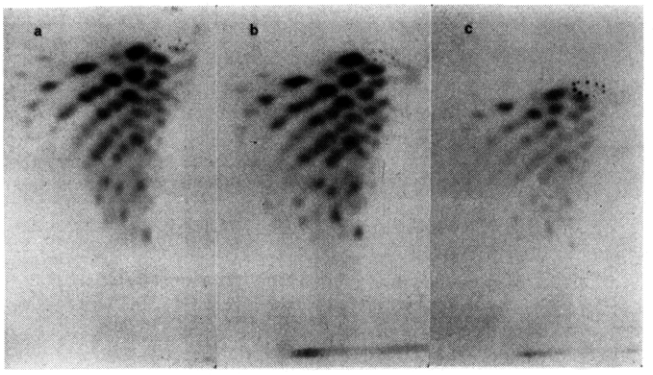

FIGURE 4: Location of "ds-oligo's in poly(A)-terminated hnRNA. [^{32}P]hnRNA was passed through poly(U)-Sepharose (Molloy et al., 1974) to select poly(A)-terminated RNA which was then separated by zonal sucrose sedimentation into large (a), medium (b), and small (c) fractions. The proportion of radioactivity in poly(A) (Molloy et al., 1974) was measured to be 3.3%, 7.8%, and 16.5% for a, b, and c, respectively, which indicates average chain lengths of 7000, 3000, and 1400 bases (Sawicki et al., 1977). Each RNA sample was boiled in H_2O and quenched in ice–water, and concentrated buffer was added to give a final concentration of 0.3 M NaCl before digestion with RNase A and T2 (see Table I). The ds-hnRNA fraction was then selected by cellulose/ethanol chromatography (Jelinek, 1977) and oligonucleotide analysis was performed after T1 digestion as described in Figure 1. After autoradiography the major large oligonucleotides (no. 1–6, see Figure 1d) were cut out and radioactivity assayed (see Table I).

were visible by fingerprint analysis (Figure 5b). However, when the mRNA was self-annealed before digestion about 2% became RNase resistant and when hybridized to DNA on filters about 2% of the mRNA became filter bound and RNase resistant. When these portions of the mRNA were subjected to fingerprint analysis, the T1 oligonucleotides characteristic of the ds-hnRNA were found to be present (Figures 5c and 5d).

To determine how many cytoplasmic mRNA molecules (in comparison with hnRNA) might contain the distinctive ds-oligo's, both poly(A)-terminated cytoplasmic and poly(A)-terminated hnRNA were purified and the total radioactivity, poly(A) content, and ds-oligo's were measured. The latter measurement was made in two ways: (1) by hybridization to DNA followed by fingerprint analysis; or (2) by self-hybridization followed by fingerprint analysis (see Figures 3, 4, and 5). The oligonucleotide spots were identified by radioautography and cut from the fingerprint, and their radioactivity was determined by scintillation counting. Table II shows that the ratio of cpm in ds-oligo's per poly(A) unit in larger hnRNA was about 1:8, whereas the ratio was at least tenfold lower in the cytoplasm (~1:80 to 1:160). While the accuracy of such measurements is probably only reliable within a factor of 2 or 3, and the content of the ds-oligonucleotides measured by first hybridizing to DNA must be at best a lower limit estimate, the conclusion is supported that the hnRNA contains considerably more ds-oligo's than does mRNA.

Distribution of ds Sequences in DNA. Nucleotide sequences in HeLa cell DNA that are complementary to the "rapidly" hybridizing portion of hnRNA have been shown to be present in all buoyant density classes of the DNA, perhaps with some preferential association with DNA of higher than average G + C content (Melli et al., 1975). We have repeated the experiments of Melli et al. and included a fingerprint analysis of the RNA that hybridized "rapidly" to the DNA taken from several different buoyant density classes. ^{32}P-labeled hnRNA that sedimented in a sucrose gradient faster than 32S pre-rRNA was collected and fragmented by alkali to oligonucleotides about 150 residues long (Jelinek et al., 1974). High molecular weight HeLa cell DNA (approximately 3×10^7 mol wt) was sedimented to density equilibrium in CsCl and fractions from the gradient were collected. DNA from each fraction of the gradient was bound to nitrocellulose filters and the ^{32}P-labeled hnRNA was hybridized to each filter. An equal aliquot of each buoyant density gradient fraction was used so that the amount of DNA per filter reflected the concentration of DNA per gradient fraction. An equal amount of the alkali fragmented, ^{32}P-labeled hnRNA was added to each filter and hybridized for 16 h, and RNase resistant hybrids were determined (Figure 6a). While there was some tendency of the DNA in the higher and lower density classes to hybridize larger relative amounts of the RNA compared with the fractions

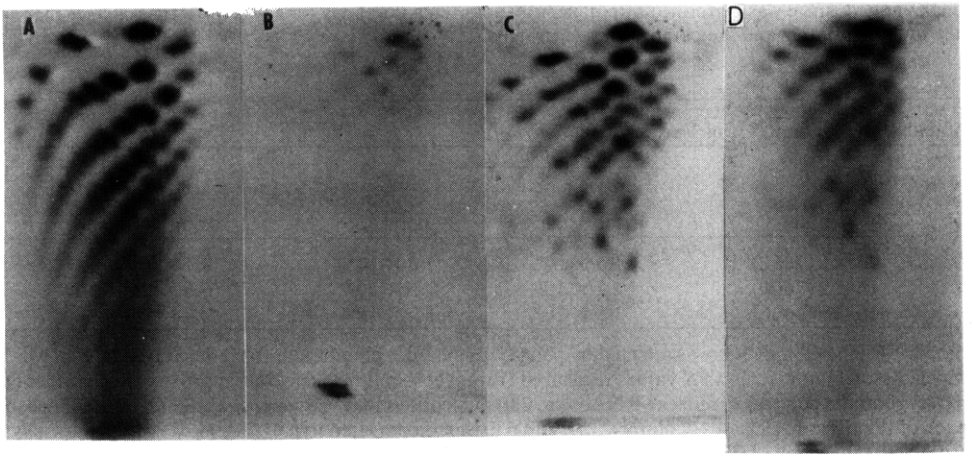

FIGURE 5: Nature of ds-oligo's in mRNA. ^{32}P-labeled polyribosomal mRNA was prepared as described from 4×10^8 cells (Experimental Procedures). Sample A was subjected to T1 digestion and oligonucleotide analysis without further fractionation. Sample B was boiled, quenched, and digested with RNases A and T2 (as in Figure 1 for hnRNA); the small amount of RNase resistant RNA (<0.1% of starting sample) was selected by cellulose/ethanol chromatography and then subjected to T1 oligonucleotide analysis as described in Figure 1. Sample C was hybridized to DNA (10 filters each bearing 50 μg of HeLa cell DNA; mRNA equivalent to 1×10^8 cells) for 24 h, RNase treatment on filters was as described in Figure 1, and hybrid RNA was eluted and subjected to T1 oligonucleotide analysis. Sample D was self-annealed (mRNA from 10^8 cells was self-hybridized in 2 × SSC, 65 °C in 0.1 mL for 20 h) and then digested with RNases A and T2 as described in Figure 1); the RNase resistant fraction was selected by cellulose/ethanol chromatography and subjected to T1 oligonucleotide analysis.

TABLE II: Content of ds Oligonucleotides in Poly(A)-Terminated hnRNA and mRNA.[a]

	total cpm	cpm in poly(A)	cpm in hybrid	cpm in ds-oligo's	ds-oligo's/ poly(A)
hnRNA, hybrid to DNA	8.7×10^6	2.5×10^5	3.6×10^5	6060	1/8
hnRNA, hybrid to DNA	1.5×10^6	4.9×10^4		1080	1/9
mRNA, self-annealed	4×10^6	4.0×10^5	7×10^4	1250	1/106
mRNA, annealed with excess hnRNA	4×10^6	4.0×10^5	9×10^4	1850	1/75
mRNA, hybrid to DNA	18×10^6	1.6×10^6	3.3×10^5	3200	1/157

[a] The hybridized RNA samples were subjected to oligonucleotide analysis and the total radioactivity in ds-oligo's 1-5 (see Figure 1) was assayed. Poly(A) content was measured on a small sample of the starting material. The ratio of ds-oligo's/poly(A) was calculated assuming (a) 50 nucleotides for the total sequences of oligonucleotides 1-5 (Jelinek, 1977; Robertson et al., 1977) and (b) a length of nuclear poly(A) of 235 and cytoplasmic poly(A) of 150 for mRNA labeled for 4 h (Sawicki et al., 1977); sample calculation for the first entry is $(6060/50)/((2.5 \times 10^5)/235) = 1/8$.

containing the majority of the DNA, in agreement with the result of Melli et al. (1975), there was less of a tendency for distinct peaks ("clustering") of DNA of increased hybridizing potential in our experiments. Filters from three regions of the gradient were pooled and the hybridized RNA recovered, digested with T1 RNase, and fingerprinted (Figures 6b-d). All three fingerprints showed the diagnostic patterns of ds-oligo's and were similar to the fingerprint of rep-hnRNA recovered from nonfractionated DNA (see Figures 1b and 1c). Figure 6 gives the results for DNA from three different density classes; other experiments performed with RNA hybridized to DNA from five different density classes gave similar results. Thus, DNA complementary to the ds-oligo's is present in all density classes.

Rates of Reassociation of ds-hnRNA and rep-hnRNA with DNA. An attempt was made to assess further the similarity of the ds-hnRNA and the rep-hnRNA by determining whether they are transcribed from equally repetitious DNA sequences. ^{32}P-labeled hnRNA was prepared, the ds-hnRNA fraction was collected after nuclease digestion, and the rep-hnRNA fraction isolated after hybridization to HeLa cell DNA. These two samples were then boiled, quenched, and incubated with DNA either in solution or immobilized on nitrocellulose filters. In addition, a portion of each of the RNA samples was incubated without any DNA to determine the portion that could self-hybridize and become RNase resistant. The amount and concentration of RNA and DNA, if present, in each hybridization reaction were the same as in all others so that the hybridization efficiency of the DNA on filters and in solution could be compared. Figure 7 shows the results of two separate experiments. Three observations were noted: (1) Both the rep-hnRNA and the ds-hnRNA hybridized at similar rates but over a broad C_0t range from 0.1 to about 100, the "intermediate" range of reassociation rates for mammalian DNA (Britten et al., 1974). (2) For the experiments shown in Figure 7a, the efficiency of hybridization to DNA on filters was higher than that to DNA in solution. In several experiments the efficiency of hybridization to the filter-bound DNA was always at least as high as, or higher than, that when the DNA was present in solution in spite of the generally accepted assumption that filter hybridization proceeds at a slower rate for most RNA:DNA reactions (Britten et al., 1974). (3) Both RNA samples showed the ability to form extensive base pairs in the absence of DNA, although the DNA enhanced significantly the rate of annealing. T1 fingerprints of the RNA hybridized at each C_0t value showed all the ds-oligos no. 1-6 as the most prominant larger oligonucleotides, even when the RNA was stripped of sequences that hybridized at lower C_0t values by previous hybridization to DNA (data not shown).

Discussion

These results lead to several conclusions about hnRNA sequences. (1) A large portion of hnRNA (as much as 27% of the larger molecules) is composed of nucleotide sequences that derive from repetitious portions of the DNA and that have the potential to form RNA:RNA duplexes (ds-hnRNA). Furthermore, all of the sequences in this repetitious fraction have a basic oligonucleotide similarity because ds-hnRNA and rep-hnRNA (isolated by RNA:DNA hybridization to presumably repetitious DNA) have similar if not identical oligonucleotide fingerprints. (2) These distinctive ds RNA oligonucleotides, ds-oligo's, are present within poly(A)-containing hnRNA molecules but the shortest hnRNA and the mRNA molecules have very much less (ca. 7%) of the ds-oligo's per molecule as do the longer hnRNA molecules. (3) All buoyant density classes of DNA can form hybrids with the ds-hnRNA and thus these sequences do not appear to be restricted to "satellite" regions (i.e., multiply-repeated, clustered, simple sequence regions of the genome; Britten & Kohne, 1968). Rather they must be dispersed throughout the genome as is thought to be true for most of the "intermediate repeat" fraction of DNA (Davidson & Britten, 1973).

The main issue raised by these experiments concerns the nature of DNA sequences that have been termed "intermediate repeat" DNA by the techniques used to measure reassociation kinetics of denatured DNA (Britten & Kohne, 1968). The "intermediate repeat" sequences are thought to represent DNA sites repeated from about 10 to perhaps 10 000 times in the genome and have been thought of as representing different "families" (Davidson & Britten, 1973). It is clear that hnRNA molecules represent transcripts from long stretches of the genome and that segments transcribed from repetitious DNA are contained within at least some of them (Darnell & Balint, 1970; Davidson & Britten, 1973). Furthermore, it is assumed that the repetitious DNA transcribed into hnRNA is at least in part "intermediate repeat" DNA (see Figure 6 and Smith et al., 1977). The results described here and elsewhere (Jelinek, 1977; Robertson et al., 1977) show that there is a close sequence relationship between the "rapidly" hybridizing hnRNA or rep-hnRNA, the "snap-back" DNA, and the ds-hnRNA. Based on T1 oligonucleotide analyses, one dominant set of repetitious oligonucleotides is present in all the hybridized (RNA:DNA and RNA:RNA) samples. Therefore, perhaps all "intermediate repeat" DNA is related to the inverted repeat DNA and contains a basic oligonucleotide similarity with some sequence divergence from site to site in the genome. The complementary segments of these inverted repeated sequences might be either immediately contiguous or removed from one

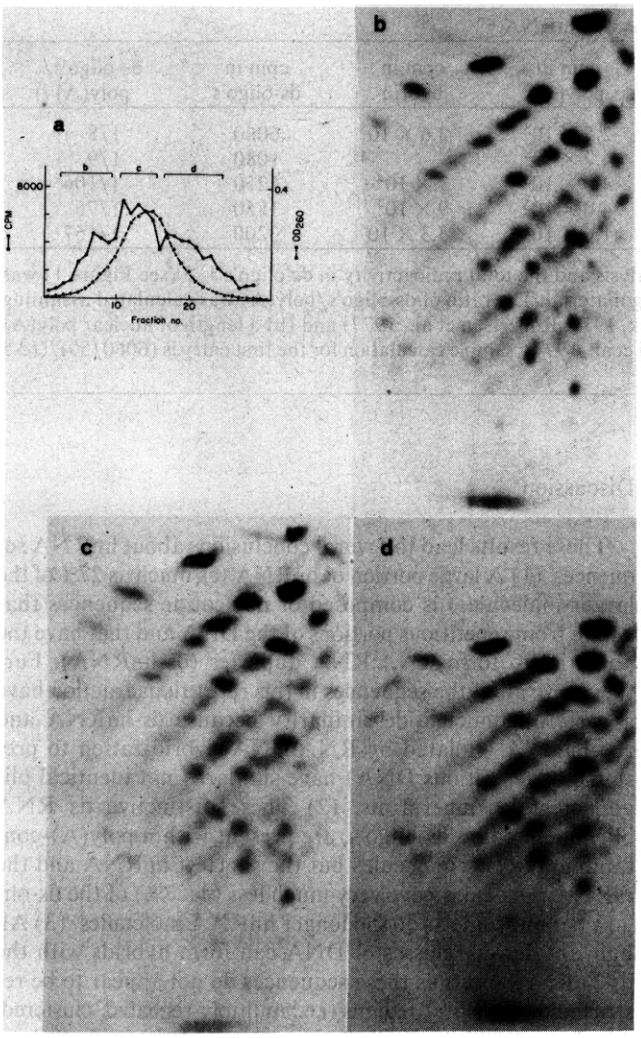

FIGURE 6: Density of DNA complementary to rep-hnRNA. HeLa cell DNA (125 μg, mol wt ca. 20×10^6) was sedimented to equilibrium in CsCl by mixing it with 56.5% (w/w) CsCl in 0.01 M Tris-HCl (pH 7.6) and centrifuging at 31 000 rpm in the Spinco 60 Ti rotor for 96 h at 20 °C. Fractions were collected by tube puncture and the optical density at 260 nM of each density fraction was determined. One-third of each fraction was denatured with alkali and adsorbed to nitrocellulose filters. ^{32}P-labeled hnRNA (5×10^5 cpm; specific activity approximately 10^6 cpm/μg) was hybridized to each filter and RNase resistant hybridization determined by Cerenkov radiation. The hybridized RNA was then eluted, digested with RNase T1, and fingerprinted. (a) A_{260} and RNase resistant RNA hybrid profile across the CsCl gradient. (b–d) T1 fingerprints of the hybrid RNA from pooled fractions indicated in a.

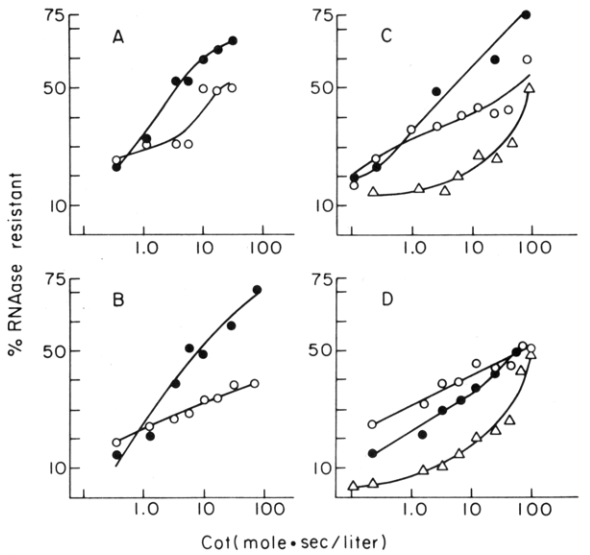

FIGURE 7: Hybridization of ds-hnRNA and rep-hnRNA to DNA on nitrocellulose filters or in solution. [^{32}P]hnRNA was fractionated as described in Figure 1a to yield ds-hnRNA or as in Figure 4c to yield rep-hnRNA. The two samples were then hybridized either to DNA bound to 2-mm nitrocellulose filters in microfilter wells (maximum capacity 0.1 mL) or to DNA in solution according to the time × concentration (C_0t) indicated. Hybridization was scored as RNase resistant, radioactive RNA. Self-annealed samples were identical with solution hybridization samples except DNA was omitted. Hybridization was at 70 °C in 0.01 M tris(hydroxymethyl)methyl-2-aminoethylsulfonic acid, pH 7.0, 0.3 M NaCl, 0.2% sodium dodecyl sulfate, 0.01 M EDTA. Data from two experiments are shown; A and B are from one experiment and C and D from the other. (A and C) Hybridization of ds-hnRNA; (B and D) hybridization of rep-hnRNA. (●) Filter hybridization; (○) solution hybridization; (△) self-annealing.

another by variable lengths of "turnaround" regions (Wilson & Thomas, 1974), i.e., unpaired sequences in the loop. If the inverted repeated sequences were immediately contiguous, they would then appear in the "snap-back" fraction; if the two inverted repeated elements were separated from one another by a long noncomplementary "turnaround" region, then after the DNA was sheared the two elements would appear in the "intermediate repeat" fraction. Such sequences could be responsible for the increased recovery of apparent ds-hnRNA in hnRNA that was self-annealed prior to RNase digestion (Federoff et al., 1977; Figure 2). The function of the ds-hnRNA or the rep-hnRNA is unknown. If these sequences do have a function in the RNA, they might serve to fold it correctly during various posttranscriptional processing events, some of which have been recently suggested to include specific cleavage and "splicing" of the RNA transcribed at noncontiguously located sites in the genome (Berget et al., 1977; Chow et al., 1977; Klessig, 1977; Breathnak et al., 1977, Hsu & Ford, 1977).

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In Vitro Reconstitution of Calf Brain Microtubules: Effects of Macromolecules†

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ABSTRACT: A comparative study has been carried out of the ability to reconstitute into microtubules of tubulins prepared by the Weisenberg and the cycle procedures. It was found that further purification of cycle tubulin by phosphocellulose chromatography made its ability to polymerize identical with that of Weisenberg tubulin. By adding to either tubulin the isolated proteins which copurify with tubulin in the cycle procedure, it is possible to reduce their critical concentrations of microtubule formation to a value identical with that of cycle tubulin. It was demonstrated quantitatively that the effect of these nontubulin proteins could be mimicked by a variety of polycationic molecules, the most effective one being poly(L-lysine). A possible mechanism is described by which growing microtubules could be stabilized by subsequent addition of the nontubulin proteins. The conclusion is drawn that, at present, it is not known whether the proteins which copurify with tubulin are specific components of the microtubule system, or simply artefactually coprecipitated impurities.

Since the initial report by Weisenberg (1972) of his discovery of conditions which lead to the in vitro self-assembly of brain tubulin into microtubules, a vast literature devoted to this subject has appeared. The aims of the various studies have been the elucidation of the mechanisms of self-assembly and of the regulation of the assembly process by metabolic effectors and drugs. The material used in most of these studies was either partially purified protein or crude homogenate of brain protein (Borisy & Olmsted, 1972; Olmsted & Borisy, 1973, 1975; Shelanski et al., 1973; Gaskin et al., 1974; Kirschner et al., 1974; Weingarten et al., 1975; Erickson, 1974; Dentler et al., 1975). The polymerization-depolymerization cycle procedure (Shelanski et al., 1973) has been the method of choice for preparing tubulin. This method in its various versions yields preparations which are 75-85% pure by the criterion of sodium dodecyl sulfate gel electrophoresis (Kirschner et al., 1974; Weingarten et al., 1975; Erickson, 1974; Borisy et al, 1974; Murphy & Borisy, 1975; Murphy et al., 1977), the remainder being a mixture of proteins, the molecular weights and amounts of which are a function of the exact conditions of preparation employed by the investigators. The observation that these nontubulin protein components coprecipitate in a constant ratio with tubulin through repeated cycles of assembly-disassembly has been used as a basis for the conclusion that these components are proteins specifically associated with microtubules rather than contaminants (Borisy et al., 1974, 195; Murphy & Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1975, 1976). Murphy & Borisy (1975) have reported that microtuble formation is promoted by heavy molecular weight components, while Weingarten et al. (1974, 1975) have concluded that dimeric tubulin is totally devoid of the ability to form microtubules and that the in vitro assembly of microtubules required the presence in stoichiometric amounts of nontubulin proteins with molecular weights considerably lower than those described by Murphy & Borisy (1975), which are found in their preparations and which they named "τ factor". In fact, tubulin dimers (5.8 S), purified by the cycle method followed by gel column chromatography, have been described repeatedly as being incapable of polymerization into microtubules by themselves (Kuriyama, 1975; Kirschner & Williams, 1974; Keates & Hall, 1975). Sandoval & Cuatrecasas (1976) compared by gel filtration chromatography tubulins prepared by various procedures (Weisenberg

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