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LIMITED NUCLEASE DIGESTION OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN IN NUCLEI

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SUMMARY: We have used limited nuclease digestion of nuclei to probe the structure of nuclear ribonucleoprotein (nRNP). Analysis of [^{3}H]uridine-labeled heterogeneous nuclear RNA isolated from nuclease digested nuclei revealed preferential generation of discrete bands of RNA ranging in size from 1.5 x 10^5 to 6 x 10^5 daltons. The nuclease digestion pattern of nRNP differed from the nuclease digestion pattern obtained with chromatin in that the RNA bands generated in these experiments were transient, appearing only early in the course of digestion, and no stable nRNP monomer size was evident. Therefore, although nRNP may be organized in a regular configuration, nRNP structure differs considerably from the repeating subunit structure of chromatin.

Several models for the organization of complexes containing hnRNA* have been proposed (for review see 1). Samarina et al. (2) isolated nRNP complexes in the form of 30S RNP particles and polymers thereof. They proposed the informofer model, suggesting that globular protein particles are arranged tandemly along and encompassing hnRNA strands. The isolation of 30-40S particles containing protein and hnRNA has led investigators to elaborate on the informofer model, proposing that RNA and protein are organized in nRNP as monoparticles which are linked together in a repeating subunit structure (3-8). It was suggested (7) that nRNP structure may be homologous to the organization of histones and DNA in chromatin nucleosomes.

The informofer model has recently been challenged. Studies by Stevenin and co-workers (9-13) have revealed the complexity and heterogeneity of RNP particles with regard to size and protein composition. Based on electron microscopic evidence, Malcolm and Sommerville (14) stated "that the ribo-

*Abbreviations used are: DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; hnRNA, heterogeneous nuclear ribonucleic acid; hnRNP, heterogeneous nuclear ribonucleoprotein; nRNP, nuclear ribonucleoprotein; RNA, ribonucleic acid; RNP, ribonucleoprotein; SDS, sodium dodecyl sulfate.

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nucleoprotein particles may not be entirely discrete in their natural state... the ribonucleoprotein particles merely being local condensates".

Much of the evidence for the subunit structure of chromatin has been derived from studies in which nuclei were digested briefly with nuclease (15-19). In those experiments, the spacer regions linking the repeating subunits were preferentially attacked by nuclease, releasing nucleosome monomers, dimers and polymers. In this report on limited nuclease digestion of hnRNP in nuclei, the data indicate that some regions of the RNA are similarly more accessible to nuclease than others. However, the results do not support the conclusion that there is a uniform repeating subunit structure of hnRNP analogous to chromatin structure.

MATERIALS AND METHODS:

Cell culture. HT-29 human colon carcinoma cells were grown in minimum Eagles' medium containing 15% fetal calf serum, as previously described (20). These cells were used 4-6 days after plating and were in log phase.

Incubation of nuclei with nuclease: nRNP studies. Actinomycin D (0.04 μg/ml) was added to log phase culture to suppress ribosomal RNA synthesis and methylation (21) in these cells (20, 22). Thirty minutes later, [^{3}H]uridine (5 μCi/ml, 30.2 Ci/mmol, New England Nuclear) was added, and incubation continued for an additional 60 min. The cells were then scraped into ice cold Hank's balanced salt solution, and lysed by vortexing in cold RSB (hypotonic buffer: 0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris, pH 7.0) containing 0.1% Triton X-100. Nuclei were pelleted at 1000 xg for 5 min, and were washed once in 5 ml of Buffer A (5 mM sodium phosphate, pH 6.8, 25 μM $CaCl_2$, 0.25 M sucrose) for subsequent incubation with staphylococcal nuclease. Nuclease digestions were carried out at 37°C.

Staphylococcal nuclease (Worthington) was added to nuclei in Buffer A to a final concentration of 1 μg/ml or 10 μg/ml. Aliquots were removed at timed intervals and EDTA added to a final concentration of 1.25 mM to stop the reaction. Sodium acetate was added to a final concentration of 10 mM, SDS was added to 0.5%, and RNA extracted by the hot phenol-SDS procedure described by Soiero and Darnell (23). The extracted RNA was precipitated by the addition of 2 volumes of ice cold 95% ethanol and left overnight at -20°C.

Gel electrophoresis. 1) 99% Formamide, 4% polyacrylamide gels were prepared and run according to the technique of Maniatis et al. (24). Cylindrical (12 cm) gels were run at 150 V until the bromophenol blue tracking dye migrated 9.5 cm. All gels were stained for 30 min in 0.1% methylene blue, 10% acetic acid, and destained overnight in H_2O. Standard markers for the gel were liver cytoplasmic RNA, containing 28S, 18S, 7S, 5S, and 4S RNA. A plot of the log relative mobility of the standards (cm migrated/cm bromophenol blue migrated) vs. the log molecular weight was linear for the five standards.

The sample gels were sliced into 3 mm fractions, dissolved in 0.3 ml of 30% hydrogen peroxide overnight at 60°C, and counted in 10 ml Aquasol (New England Nuclear) containing 5% H_2O in an Intertechnique scintillation counter. Efficiency was monitored by channel ratio using an external standard.

The size of RNA in the gels was estimated from the standard curve. Weight averages were calculated by the method of Vournakis et al. (25), using the formula:

$$\bar{M}_w = \frac{\sum_i (CPM)_i \cdot (M_i)}{\sum_i (CPM)_i}$$

where $\bar{M}_w$ is weight average molecular weight; $(CPM)_i$, the radioactivity of gel slice i; and M_i, the molecular weight of the RNA in gel slice i.

2) 5% Polyacrylamide, 7 M urea 10 cm slab gels were prepared according to the method of Maxam and Gilbert (26) and run at 100 V for 3 hr. Fluorography of the [^{3}H]-labeled RNA run in these gels was by the method of Bonner and Laskey (27). Standards were whole cell RNA from HT-29 cells labeled for 5 hr with [^{3}H]uridine.

RESULTS: Utilization of limited nuclease digestion to probe nRNP structure is complicated by the rapid degradation of nuclear RNA by endogenous nuclease. Figure 1 (closed circle) shows that incubation of isolated nuclei at 37°C in the absence of added nuclease resulted in a marked decrease in the weight average molecular weight of the hnRNA. The addition of 1 μg/ml staphylococcal nuclease (Figure 1, open circles) produced a modest increase in the rate of degradation, and this rate was further increased as the enzyme concentration was raised to 10 μg/ml (open triangles). Thus, at high enzyme concentrations and short incubation, the effect of endogenous nuclease was minimized relative to the cleavage initiated by the exogenous nucleases.

The question we next addressed was whether all regions of the polynulceotide were equally accessible to the enzyme. That is, whether or not discrete size classes of RNA were generated early during the course of digestion. To answer this question, cells were labeled with [^{3}H]uridine in the presence of actinomycin D under conditions in which the synthesis and methylation of ribosomal RNA are preferentially suppressed in these cells (Methods). The nuclei were isolated and incubated with 10 μg/ml staphylococcal nuclease. At various stages of digestion, the labeled RNA was extracted and analyzed by electrophoresis in slab gels followed by fluorographic visualization of radioactivity (27). Figure 2a shows the patterns obtained. At 4 and 5 minutes of digestion, a series of discrete bands, ranging from 1.5 to 6 x 10^5 daltons, were seen. Although the precise

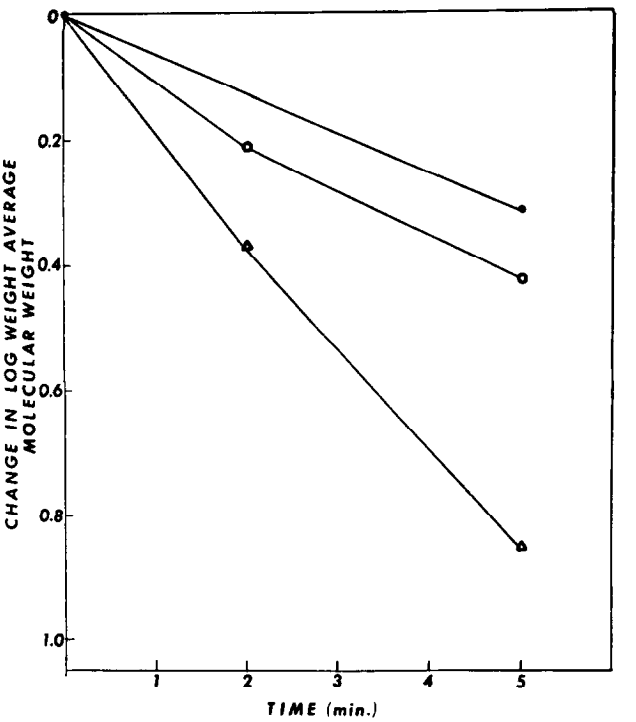

Fig. 1 - Changes in log weight average molecular weight of hnRNA during incubation of nuclei. Cells were pretreated with 0.04 µg/ml actinomycin D, then incubated for 60 min with 5 µCi/ml [^{3}H]uridine. Isolated nuclei were incubated at 37°C for 0 to 5 min:

● - without the addition of enzyme (endogenous nuclease activity)

0 - with 1 µg/ml staphylococcal nuclease

Δ- with 10 µg/ml staphylococcal nuclease

RNA was extracted, analyzed on formamide gels, and the weight average molecular weight of the RNA at various time points was calculated by the method of Vournakis, et al. (25) (see Methods).

timing of the digestion and prominence of the bands varied in different experiments, similar results were seen in 2 other independent experiments (Figure 2b and c). In each experiment, a series of digestion times was analyzed, but only one time point in each is shown. The critical control for these experiments is shown in Figure 3. Here, cells were labeled for 1 hour with [^{3}H]uridine, and nuclear RNA isolated by phenol extraction (Methods). The deproteinized RNA was then digested under conditions identical to those used for the digestion of nuclei, although for shorter

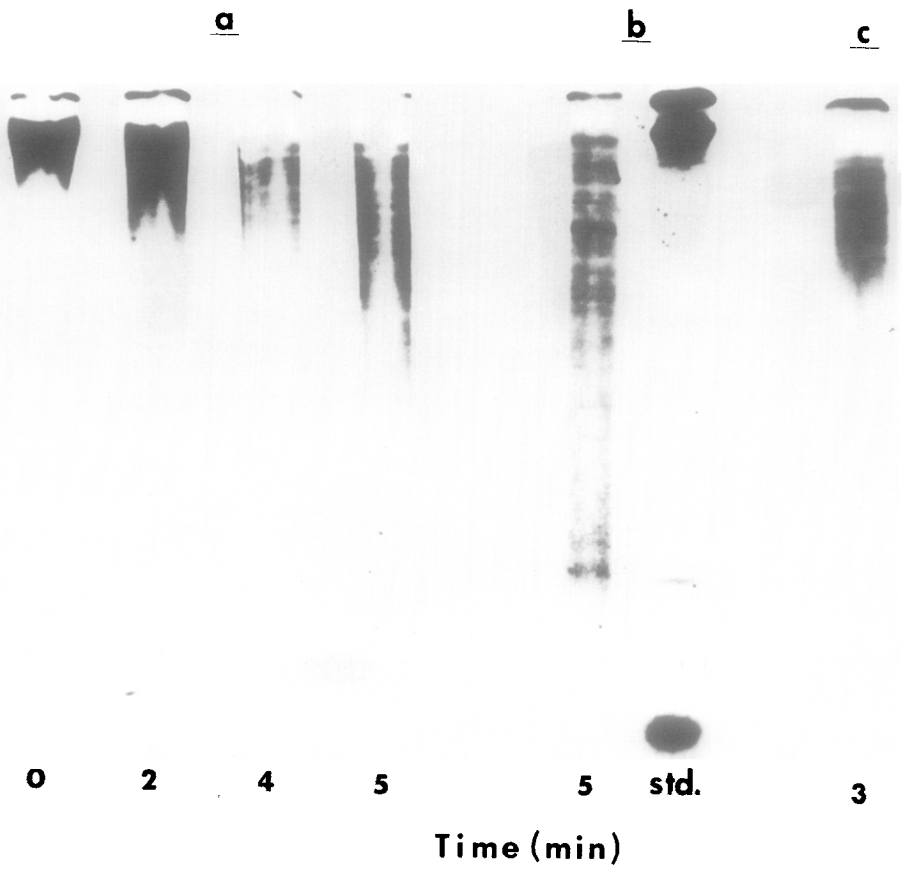

Fig. 2 - Polyacrylamide gel electrophoresis of hnRNA from nuclei digested with staphylococcal nuclease; three independent experiments (a-c). In each, discrete bands of RNA were detected. Nuclei were prepared as in Figure 1, and incubated with 10 µg/ml staphylococcal nuclease for: (a) 0, 2, 4 and 5 min, (b) 5 min, and (c) 3 min. RNA was extracted and analyzed on 5% polyacrylamide 7M urea gels (26) and radioactivity was visualized by fluorography (27). Standards were run with each gel. A representative standard gel (std.) is shown for experiment (b); from top to bottom, bands represent 28S, 18S, 7S and 4S RNA, respectively.

times. The pattern of radioactivity observed following analysis of RNA on gels indicated that the RNA was degraded as incubation with nuclease preceeded, but in this control experiment no discrete bands appeared in the high molecular weight region of the gel. Similar results were obtained in three independent experiments (two experiments are shown). In addition, although incubation of nuclei at 37°C in the absence of added enzyme led to slower degradation of the labeled RNA by endogenous nuclease (Figure 1), no bands were seen at

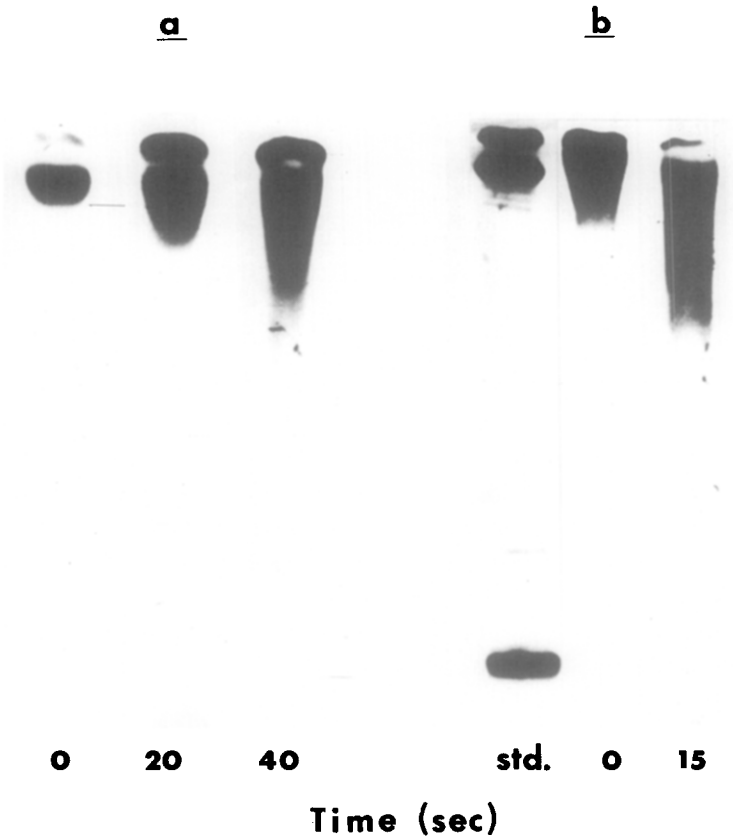

Fig. 3 - Nuclease digestion of deproteinized RNA; two indepedent experiments (a and b). RNA was isolated by the hot phenol-SDS extraction method of Soiero and Darnell (23) and incubated with 10 µg/ml staphylococcal nuclease for: (a) 0 sec, 20 sec, 40 sec, and (b) 0 sec, 15 sec. RNA was re-extracted and analyzed on polyacrylamide gels as in Figure 2. Standards were run with each gel. A representative standard gel (std.) is shown for experiment (b); from top to bottom, bands represent 28S, 18S, 7S and 4S RNA, respectively.

1, 2, 3, 4 or 5 minutes in the absence of exogenous enzyme (not shown). It is therefore clear that the cleavage pattern of the hnRNA seen in Figure 2 is dependent on a structural organization of the RNA in the nucleus which is lost upon isolation of the RNA.

DISCUSSION: Nuclease digestion of nuclei containing [^{3}H]uridine labeled hnRNA led to the generation of specific size classes of RNA. The appearance of discrete bands in the high molecular weight region of the gel was reproducible in three experiments. The results demonstrate that some sites in nRNP are

relatively more accessible to nuclease, and, since the cleavage pattern is not seen in the isolated RNA, the digestion pattern depends on a structural organization of the hnRNA in situ which is lost upon isolation. It is unlikely that the bands represent contamination by cytoplasmic messenger RNA since nuclei prepared by detergent treatment were free of cytoplasmic contamination as determined by electron microscopy (20). The bands are never seen at 0 time of digestion, indicating they are only generated by cleavage of higher molecular weight RNA. The question arises as to why such a complex pattern is seen rather than a more simple pattern as predicted by the subunit model of nuclear RNP structure (1-8). At least two explanations are possible. First, evidence has been presented for size heterogeneity of nRNP monoparticles (13). Second, nRNP monoparticles may not be regularly spaced along the RNA, or may be spaced differently along different sequences. Nevertheless, our results indicate that the analogy drawn between chromatin structure and nRNP structure may not be strictly correct, as has already been suggested by others (1, 12-14).

It should be noted that the discrete size classes of RNA molecules appear only transiently, early in the digestion, and further incubation with nuclease results in a smear of radioactivity in the lower molecular weight region of the gel. The digestion of DNA in chromatin is similar to that of RNA in nRNP at early stages of incubation in that discrete size classes are generated (15-19, and our data, not shown); however, upon further incubation of chromatin, discrete lower order DNA structures accumulate. Therefore, the RNA may be more accessible in nRNP than DNA is in nucleosomes. This has also been demonstrated by other studies; RNA isolated from 30-40S monoparticles is usually extensively degraded (2, 4, 28). In addition, we have previously reported that only about 10% of the hnRNA in nRNP is protected from nuclease and can be isolated as fragments approximately 30 nucleotides in length (29).

In summary, we have presented evidence suggesting that protein is associated with RNA in a specific manner in nRNP, making some sites more accessible to nuclease than others. However, our data strongly indicate

that the nRNP structures are not as rigidly defined as the chromatin nucleosome. Indeed, a flexible and adaptable organized structure, rather that a rigid one, may be necessary for the extensive sequence editing now known to be involved in hnRNA processing.

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