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The release of 40S hnRNP particles by brief digestion of HeLa nuclei with micrococcal nuclease

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ABSTRACT

Brief digestion of HeLa nuclei with micrococcal nuclease releases monomer hnRNP particles as well as monomer and polynucleosomes. Sucrose gradient analysis of the nuclease released material reveals a series of small A260 peaks overlapping a more predominant peak in the 40S region of the gradient. Analysis of the proteins, DNA, and RNA in successive gradient fractions has confirmed that the smaller peaks are monomer and polynucleosomes, and that the larger peak is 40S hnRNP. Like 40S particles isolated by low salt extraction or by sonication, the nuclease released particles are composed of rapidly labeled RNA associated with a group of non-histone proteins the most predominant of which are the 32,000-44,000 MW proteins previously identified as core hnRNP proteins. These results provide further evidence that 40S hnRNP particles exist as discrete structural components of larger in vivo ribonucleoprotein complexes.

INTRODUCTION

In eukaryotes heterogeneous nuclear RNA is associated with a specific subset of non-histone proteins forming a complex which may represent the site of the post-transcriptional modifications which process nascent RNA transcripts into functional messenger RNA. Although this RNA-protein complex has been studied for several years by a number of investigators, disagreement remains concerning its in vivo organization. RNA-protein complexes have been visualized in ultrastructural studies of actively transcribing non-ribosomal genes as an array of fibrils of increasing lengths, with regularly spaced globular structures about 20 nm in diameter which give the fibrils a beaded appearance (1,2). Monomer 40S hnRNP particles have been isolated from the nuclei of a variety of eukaryotic cells by a lengthy low-salt extraction procedure first described by Samarina and co-workers (3), which

favors the action of endogeneous nucleases. The release of monomer particles 21-25 nm in diameter and containing rapidly labeled RNA by this procedure is consistent with the interpretation that 40S hnRNP particles are released from the nascent fibrils of transcriptionally active genes (4,5,6).

Other investigators have employed brief sonication to release ribonucleoprotein complexes from isolated nuclei (7,8,9). Unlike the particles extracted with low salt, particles released by nuclear sonication have shown a more heterodisperse sedimentation pattern with sedimentation coefficients between 30 to 200S. While the material from the 30 to 40S region of these gradients contains the same major proteins seen in particles extracted from nuclei with low salt, the more dipersed sedimentation patterns of hnRNA, snRNA, and proteins make it difficult to determine which proteins are associated with specific subnuclear structures. Unlike previously reported results, we find that hnRNP particles released by sonication of HeLa nuclei have the same sedimentation properties and protein composition as low salt extracted particles. The major difference in the two particle preparations is in the size of the RNA component.

We now report that brief digestion of isolated HeLa nuclei with micrococcal nuclease releases monomer hnRNP particles along with monomer and polynucleosomes. Like the particles released by low salt extraction or by sonication, the nuclease released particles sediment at 40S in sucrose gradients and are complexes of newly synthesized RNA and the previously identified hnRNP proteins (4,10). The release of hnRNP by brief nuclease digestion supports the contention that these particles exist as a component of larger _in vivo_ structures which are cleaved by endogeneous nucleases during the lengthy low salt extraction procedure. In addition these findings support the contention that 40S hnRNP particles are discrete structural units which pre-exist in nuclei and are not artifacts resulting from protein-RNA rearrangements during extraction as has been suggested (11).

As a result of these findings, we point out the necessity of distinguishing between DNA-protein complexes and RNA-protein complexes when analyzing material released from nuclei by

micrococcal nuclease digestion. It is well known that this enzyme acts as a nonspecific endonuclease cleaving RNA as well as DNA (12,13). Recent publications have reported the preferential release of 8N and 16N polynucleosome structures from nuclei following brief micrococcal nuclease digestions (14,15,16). Using very similar experimental conditions, we find no evidence for the specific release of any size class of polynucleosomes and suggest that 40S hnRNP could have been mistaken for 8N polynucleosomes.

MATERIALS AND METHODS

Cell culture and nuclear isolation: Hela cells were grown in suspension culture in minimal essential medium supplemented with 5% calf serum, and harvested when the cultures reached densities of 3 to 5 x 10^5 cells per ml, to ensure that cells were in log phase growth. RNA was labeled by adding 0.25 uCi of ^{3}H-uridine (Schwarz-Mann 39 Ci/mmole) per ml of culture medium immediately after the cells were concentrated 3 to 4 times by gentle centrifugation followed by resuspension in fresh medium. Nuclei were isolated by procedures previously described (4), and the preparations were routinely checked for cytoplasmic contamination and nuclear breakage by phase contrast microscopy.

Isolation of hnRNP: Procedure I. HnRNP particles were isolated by repeated extraction of the purified Hela nuclei with STM buffer (0.1 M NaCl, 1.0 mM $MgCl_2$, 10mM Tris-HCl pH 8.0) as described previously (4). Particles were collected onto 0.2 ml cushions of 75% sucrose in STM by centrifuging the combined extracts at 166,500 x g for 2.5 hours at 4^{o}C, after which the sucrose cushions along with 0.6 mls of the STM buffer were removed from the bottom of the centrifuge tubes. The recovered material was dialyzed against STM buffer to reduce the sucrose concentration before it was analyzed on 15 to 30% sucrose gradients in STM buffer using a Beckman SW 27 rotor at 25,000 rpm for 15 hours.

Procedure II. HnRNP particles were also isolated using a sonication procedure similiar to that used by other investigators (8,9). Purified nuclei were washed in STM buffer, then resuspended in 5 volumes of STM. The nuclei were subjected to

repeated 10 second bursts of sonication until all the nuclei were broken, with the extent of nuclear breakage determined using phase contrast microscopy. Nuclear membranes, nucleoli, and large bits of chromatin were removed by centrifugation at 16,300 x g for 15 minutes. The resulting supernatant, which contained the nuclear hnRNP, was analyzed on 15 to 30% sucrose gradients as decribed above.

Nuclease Digestion: Purified nuclei were washed once in nuclease digestion buffer (80mM NaCl, 5mM Tris-HCl pH 7.5, 1mM $CaCl_2$) and resuspended in this buffer at a known concentration, usually 1 or 2 x 10^8 nuclei per ml. The nuclear suspension was preincubated at 37°C for 5 minutes before the addition of micrococcal nuclease (Worthington) at a concentration of 30 units per 10^8 nuclei. Digestion was stopped by transferring the nuclear suspension to an ice cold centrifuge tube containing a predetermined volume of 0.1 M EDTA making the final concentration 5mM EDTA. The nuclei were pelleted at 5,000 x g for 8 minutes and this first supernatant was saved on ice. The pellet was resuspended in digestion buffer with 5mM EDTA and extracted on ice for 20 minutes. The nuclei were again pelleted, and the first and second supernatants were combined. The material released by brief nuclease digestion, was analyzed on 10 to 30% sucrose gradients made in Buffer G (80mM NaCl, 10mM Na phosphate, pH 6.8, 5mM EDTA) using the SW 27 rotor at 25,000 rpms for 12 hours.

Analytical Procedures: After centrifugation the gradients were scanned at 260 nanometers using a Gilson UV column monitor and fractionated. In some cases adjacent fractions were pooled to provide larger samples for analysis.

The distribution of labeled RNA in the gradients was determined by collecting the TCA precipitable material from aliquots of each gradient fraction on Whatman GCA filters or on 3mm paper and counting the filters in Aquasol (Amersham).

To determine the DNA distribution in the gradients, successive fractions were dialyzed against distilled water and lyophilized to dryness. The samples were hydrolyzed with 0.5 M PCA and DNA per fraction was determined using the diphenylamine assay (18).

To determine the distribution of specific proteins in the gradients, the proteins of each gradient fraction were analyzed by polyacrylamide gel electrophoresis. Individual fractions were dialyzed against 0.2% SDS, lyophilized to dryness, then dissolved in SDS sample buffer. Proteins were resolved on 12% SDS polyacrylamide gels by procedures described elsewhere (19). Samples for nucleic acid gels were prepared in the same way as the protein samples, then treated with 5ug/ml Proteinase K (EM Biochemicals) immediately before they were loaded onto 10% SDS gels. The gel composition and running conditions were the same as for the protein gels. In some experiments the nucleic acid samples were resolved on nondenaturing 1.5% agarose slab gels. The gel buffer was 40mM Tris acetate pH 8.3 with 1mM EDTA, and electrophoresis was at 150 volts for six hours. The nucleic acid gels were stained with 1ug/ml Ethidium Bromide in 1mM EDTA pH 8.0 or with 2mg/ml methylene blue in 10% acetic acid, 10% methanol, and destained with water.

RESULTS

The release of 40S hnRNP by STM extraction and by sonication: Figure 1 shows a comparison of 40S hnRNP prepared by STM extraction and by sonication. Both procedures result in the release of an hnRNA-protein complex which migrates in the 40S region of a sucrose gradient (figure 1a and 1b). SDS polyacrylamide gels of the proteins in successive fractions from these two gradients (figure 1c and 1d) show essentially no differences in the protein components of the two preparations. However, the RNA components are significantly different (figure 1e and 1f). The STM extracted particles contain a population of small heterogeneous RNA molecules which appear to be quite degraded, while the RNA of the sonicated particles is much larger in size. Discrete high molecular weight bands are resolved at the top of the lanes corresponding to the 40S hnRNP peak, and there is very little RNA staining in the lower half of the gel. This finding is consistent with the hypothesis that endogeneous nucleases are involved in the release of 40S hnRNP during the lengthy STM extraction procedure, and suggests that the larger RNA fragments present in particles isolated by sonication are probably more representative

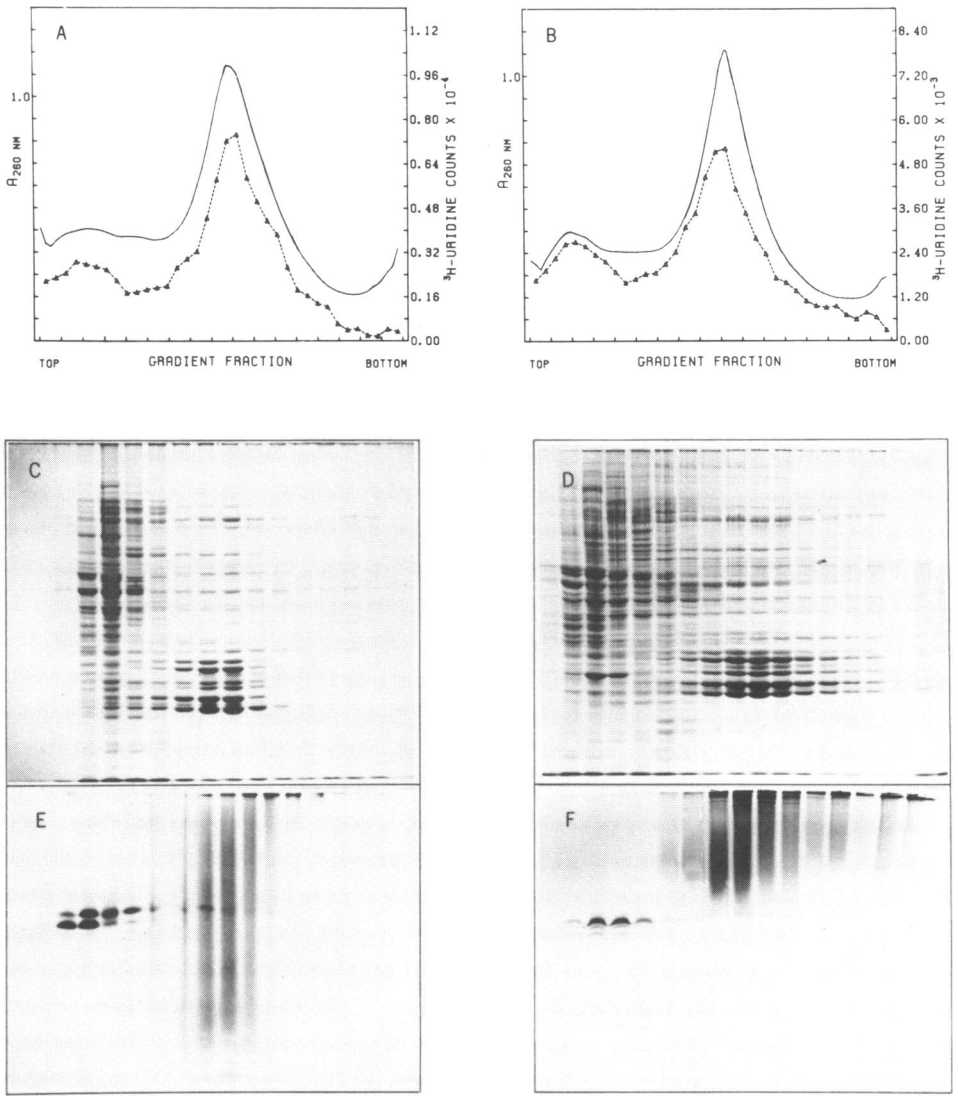

Figure 1. Comparison of 40S hnRNP particles isolated by low salt extraction (panels A, C, and E) and by sonication (panels B, D, and F). Panels A and B show the distribution of A260 material (—) and 3H-uridine counts (--) in 15 to 30% sucrose gradients. 2 to 3 x 10 HeLa cells were used for each particle preparation shown. Panels C and D, 8.75% SDS polyacrylamide gels of the proteins in successive fractions from the sucrose gradients. Panels E and F, 10% SDS polyacrylamide gels of the RNA in successive fractions from the sucrose gradients. The RNA gel is stained with methylene blue.

of the in vivo RNA of the particles.

The RNA gels in figures 1e and f also show a population of low molecular weight RNA molecules migrating just ahead of the 40S hnRNP peak. These RNA molecules appear to be a component of a second population of nuclear RNP particles similar to the snRNPs described by Lerner and co-workers (20). Based on their distribution in our gradients, we have concluded, like Lerner and Steitz (21), that these low molecular weight RNAs are not structural components of 40S hnRNP, as has been suggested by other investigators (22,23,24).

Release of 40S hnRNP by brief micrococcal nuclease digestion: HeLa nuclei were digested briefly with micrococcal nuclease, and the released material was resolved on sucrose gradients essentially as described by Butt and co-workers (14). Two types of peaks are seen when the sucrose gradients are scanned at 260 nanometers. A group of small successive peaks in the top half of the gradient are monomer and polynucleosomes of increasing number, as indicated by their sedimentation properties, their digestion kinetics, as well as analysis of their DNA and protein components (figure 2). In some preparations as many as nine successive peaks were resolved, suggesting that the gradient conditions used in these studies will resolve polynucleosome structures of up to 9N particles. Two much broader peaks, labeled A and B in figure 2, are seen in the lower half of the gradients. The faster sedimenting peak A decreases in size as digestion times are increased, suggesting that it is a precursor to some smaller, more slowly sedimenting structure. Peak B, the second broad peak, is more prominent in the gradient after 120 seconds of digestion than after either 60 seconds or 300 seconds of digestion, and it migrates with polynucleosomes 6N to 9N in length. When 40S hnRNP particles, isolated by the STM extraction or sonication procedures, are run on parallel gradients, they co-sediment with peak B (figure 2).

HnRNP particles were identified in the nuclease released material by carefully analyzing the distribution of DNA, RNA, and specific proteins in the sucrose gradients. Figure 3 shows the distribution of RNA and proteins after 120 seconds of digestion where a prominent peak of TCA precipitable ^{3}H-uridine labeled

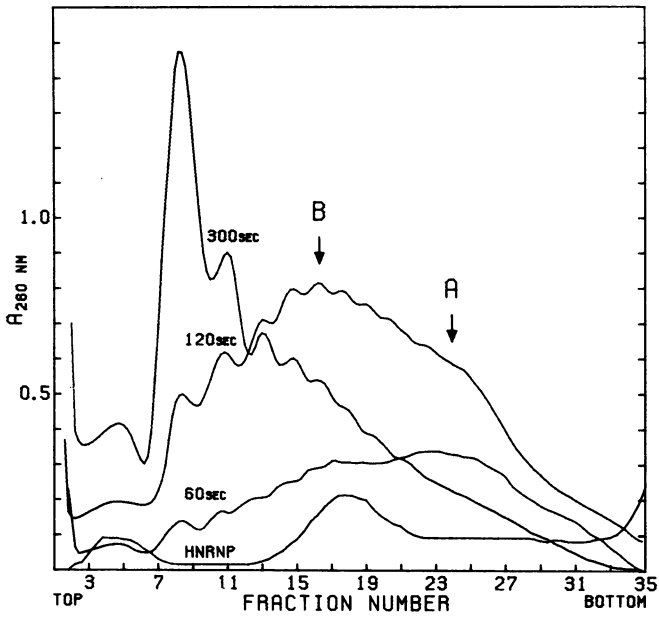

Figure 2. Sucrose gradient analysis of the material released by brief micrococcal nuclease digestion. Isolated HeLa nuclei at a concentration of 1 to 2 x 10 nuclei per ml were digested with 30 units of micrococcal nuclease per 10 nuclei for the times shown. The released material was analyzed on 10 to 30% sucrose gradients as described in materials and methods. The migration of purified 40S hnRNP in a parallel gradient is shown as a marker.

material is associated with peak B. Control experiments have shown that this material is both alkali and RNase sensitive.

The lower panel of this figure shows the proteins in successive fractions of the gradient resolved on a 12% SDS polyacrylamide gel. The distribution of nucleosome core histones H2a, H2b, H3, and H4, histone H1, and numerous nonhistone proteins can be clearly seen in this complete analysis of the protein distribution in sucrose gradients. The major protein components of monomer 40S hnRNP are seen in the gradient fractions corresponding to peak B and the rapidly labeled RNA. Comparison of the proteins of 40S hnRNP isolated by low salt extraction and by sonication with those released by brief micrococcal nuclease digestion are shown in figure 4. With the

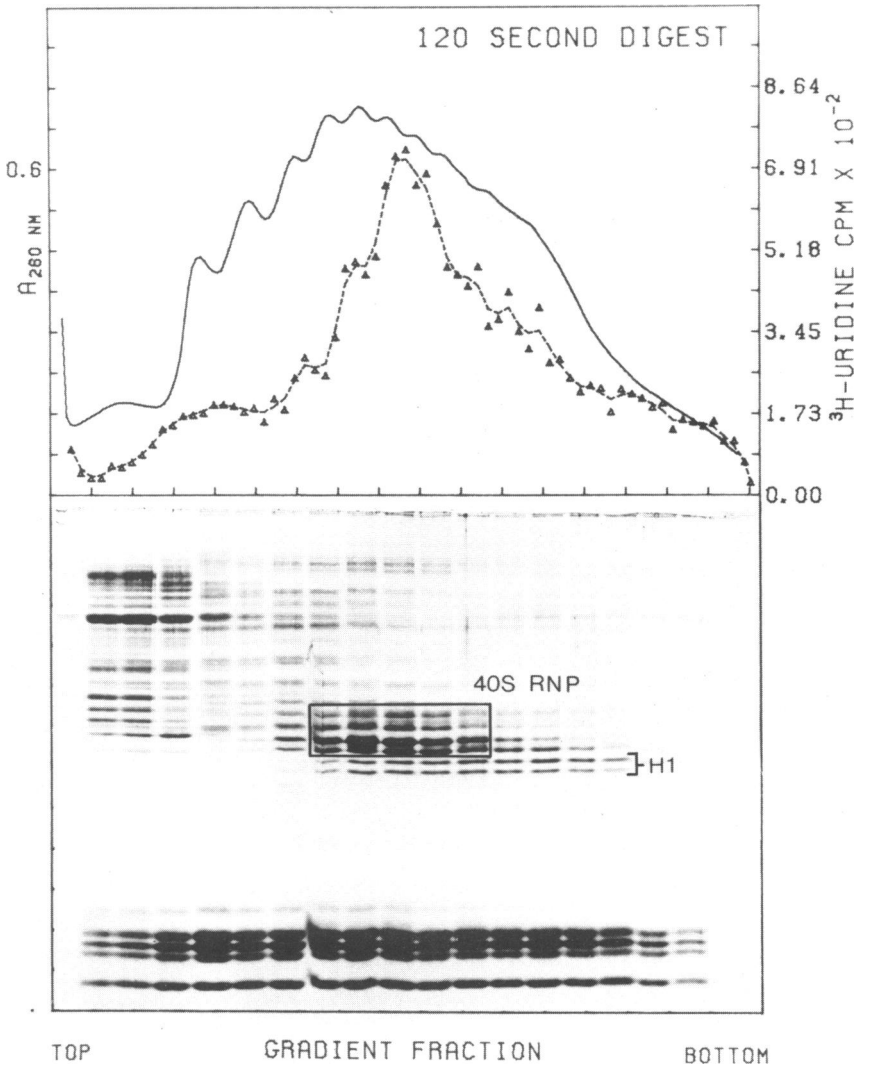

Figure 3. Analysis of the material released after 120 seconds of digestion. The top panel shows the distribution of A260 material (——) and TCA precipitable 3H-uridine counts (Δ---Δ) in a sucrose gradient of the material released by 120 seconds of digestion. The curve for TCA precipitable counts was drawn using a five point averaging function to reduce experimental noise from sampling errors. The lower panel shows a 12% SDS polyacrylamide gel of the proteins in successive fractions across the sucrose gradient.

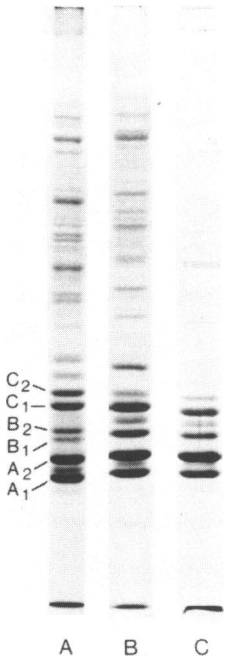

Figure 4. Comparison of the proteins from the 40S peak fractions of sucrose gradients of hnRNP particles prepared by A) STM extraction, B) sonication, C) micrococcal nuclease digestion, resolved on 8.75% SDS polyacrylamide gels. The major RNP proteins are identified.

exception of the histones, the protein compositions of the three preparations are strikingly similar, providing definitive identification of 40S hnRNP in the nuclease released material.

To further demonstrate that hnRNP particles are a significant component of the material released by brief micrococcal nuclease digestion, the distribution of DNA was determined and compared to the distribution of RNA in the same gradient. Figure 5 shows the gradient distribution of DNA in a 120 second digest determined using the diphenylamine assay. The distribution of DNA does not follow the distribution of A_{260} material demonstrating that a significant portion of the material in peak B is something other than DNA. The individual contributions of DNA and RNA to the A_{260} profile of this gradient were estimated by calculating the ratio of TCA precipitable ^{3}H-uridine counts per

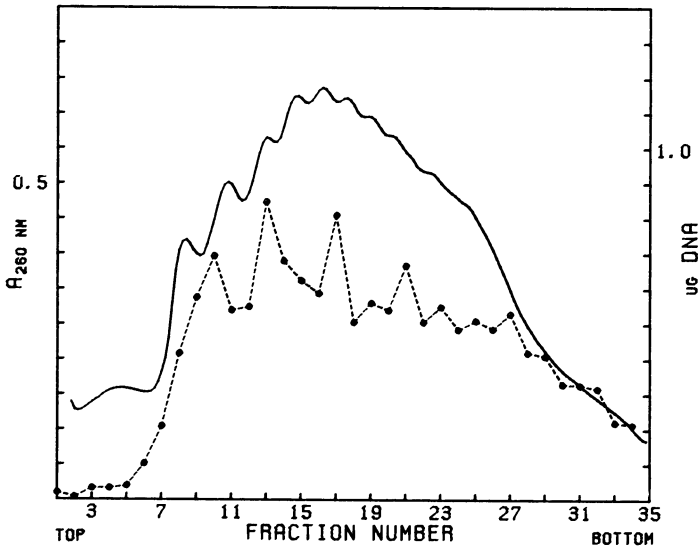

Figure 5. The distribution of DNA in a sucrose gradient of the material released following 120 seconds of nuclease digestion. The amount of DNA in each gradient fraction was determined using the diphenylamine assay.

A_{260} unit and the ratio of ug of DNA per A_{260} unit for each gradient fraction. Plots of these ratios (figure 6) show that a significant amount of the A_{260} absorbing material in the fractions of peak B is actually RNA and not DNA. Thus the total A_{260} profile, neglecting the small contribution by the proteins in the gradient, appears to be made up of a broad peak of RNA, which is overlapped by the smaller successive polynucleosome peaks. The analysis of gradients of material released after 60 and 300 seconds of digestion also shows 40S hnRNP migrating with polynucleosomes 6N to 9N in length. The release of neither 40S particles nor polynucleosomes was detected in control experiments where micrococcal nuclease was omitted from the digestion reaction, indicating that the release of both the RNA-protein complexes and the DNA-protein complexes was dependent on the presence of the enzyme.

Gels of the nucleic acid in successive sucrose gradient fractions show a typical repeat pattern with broad bands of DNA

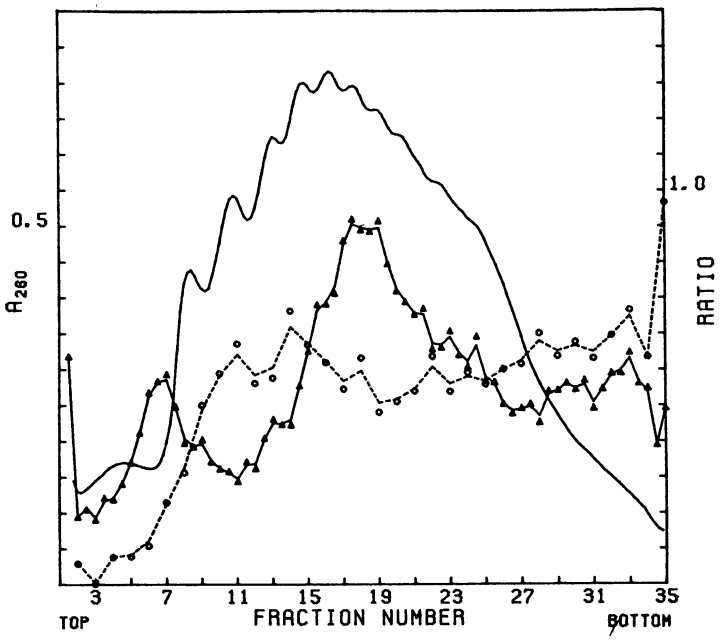

Figure 6. Relative contributions of RNA and DNA to the total A260 material resolved on a sucrose gradient. (Δ—Δ) TCA precipitable 3H-uridine counts per A260 unit; (o--o) ug of DNA per A260 unit. Aliquots from every two gradient fractions were pooled to provide sufficient samples for determining DNA per fraction. The curves were drawn using a weighted averaging function to reduce the noise from experimental sampling errors.

increasing in length corresponding to the sedimentation pattern of the polynucleosomes in the gradient (figure 7). It is not possible to draw conclusions about the size of the RNA associated with the hnRNP from these gels since the intensity resulting from the ethidium staining of double stranded DNA fragments is much greater than the intensity from its association with the RNA.

Implications for Higher Order Nucleosome Structure: It has been suggested that brief nuclease digestion results in a non-random cleavage of the chromatin in nuclei, releasing at least two predominant classes of polynucleosomes of repeat size 8N and 16N, and that the 8N and 16N structures are representative of some unique higher order structural component of chromatin (14,15,16). If this is true, when the material from such brief

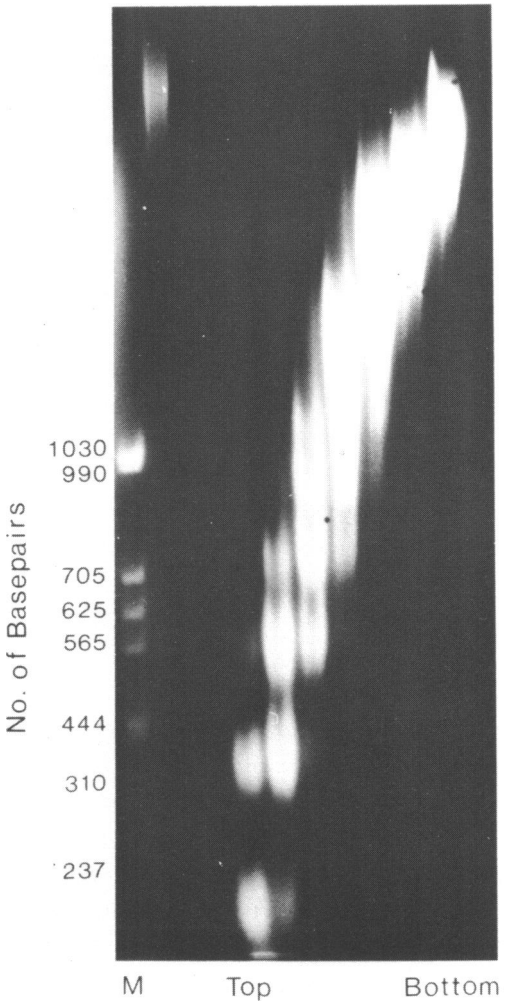

Figure 7. Agarose gel electrophoresis of the nucleic acid fragments in fractions across the sucrose gradient. HeLa nuclei at a concentration of 1 x 10^8 nuclei per ml were digested for 60 seconds with 30 units of micrococcal nuclease per 10^8 nuclei, and the nuclease released material was analyzed on a sucrose gradient. (See the gradient profile for the 60 second digest in figure 2.) Fractions were prepared for electrophoresis, then treated with 5ug per ml (final concentration) of Proteinase K immediately before they were loaded onto 1.5% agarose slab gels. The marker is E. coli plasmid ColEl digested with restriction enzymes Hae II and Hae III.

micrococcal nuclease digests is resolved on sucrose gradients, there should be a substantial increase in the amount of core histone molecules in the gradient fractions containing the 8N and 16N polynucleosomes, corresponding to the increased numbers of these particles that should be released. Other size classes of polynucleosomes should result primarily from random cleavage between nucleosomes of the 8N and 16N polynucleosome structures. On the other hand, if the nuclease cleavage is random there should be approximately equal amounts of each size class of monomer and polynucleosomes released by brief digestion, resulting in a linear distribution of core histone molecules and DNA across the gradient with an increasing slope determined by the resolution of monomer and polynucleosomes in the gradient. For these considerations it is important to remember that one 8N polynucleosome will have approximately 8 times the core histone molecules of one monomer nucleosome or 4 times that of one dimer nucleosome.

The A_{260} gradient profile and DNA distribution of the nuclease released material from HeLa nuclei digested for 90 seconds, but with only 6 units of enzyme per 10^8 nuclei, is shown in the upper panel of figure 8, and a gel of the proteins associated with each gradient fraction is shown in the lower panel. Each track of the gel, which was stained with Coomassie Blue, was scanned at 555 nanometers to quantitate the amount of histone and RNP proteins in each gradient fraction. The scans were photocopied, and the core histone peaks and RNP protein peaks were cut out and weighed on an analytical balance. The results of this quantitation are shown in figure 9. The 40S hnRNP proteins are distributed in the gradient fractions corresponding to peak B, with some trailing into lower regions of the gradient. The concentration of core histones increases down the gradient with no obvious increase in the fractions where 8N and 16N structures should be present. If one compares the absolute amounts of histone molecules in the fraction containing the monomer nucleosome peak, to the amount in fractions containing 8N or 16N polynucleosomes, one finds that the amount of histone is not enough to account for the release of even one 8N or 16N polynucleosome for each monomer released in this very brief

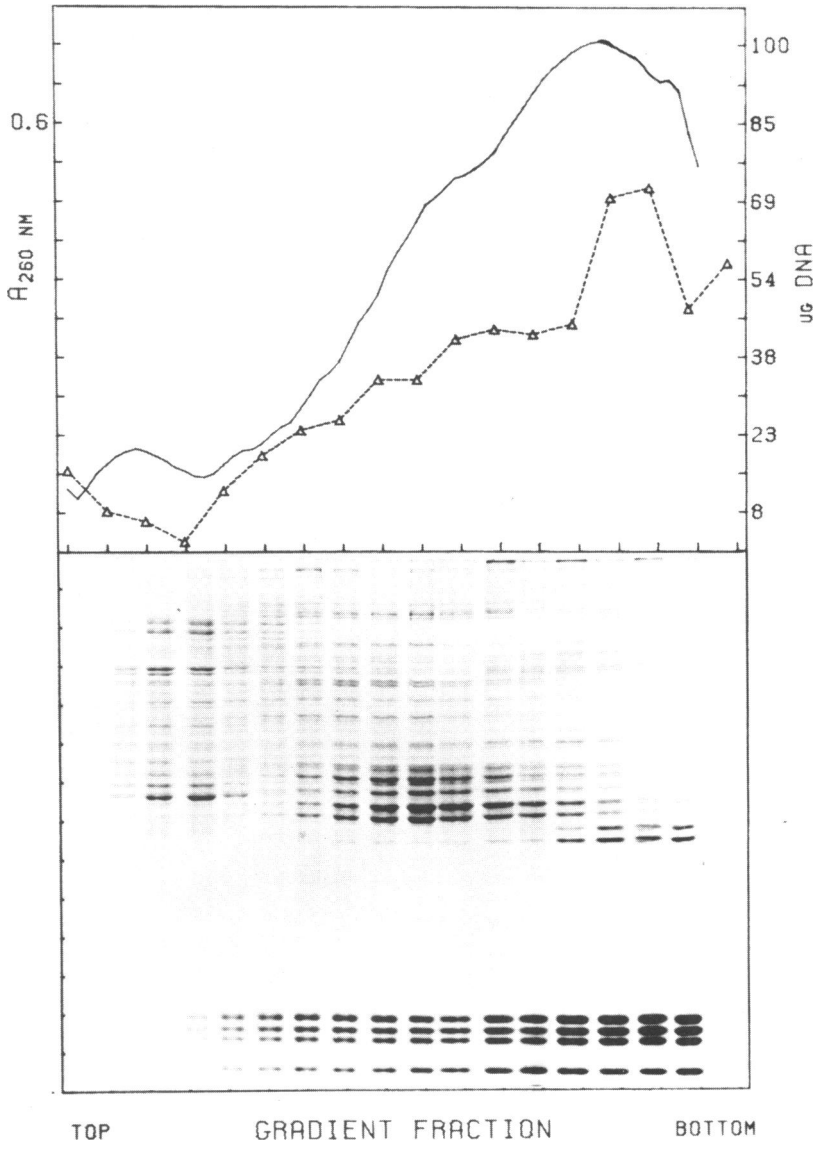

Figure 8. Analysis of a very brief nuclease digestion. HeLa nuclei at a concentration of 3 x 10^8 nuclei per ml were digested with 6 units of micrococcal nuclease per 10^8 nuclei for 90 seconds, and the nuclease released material was analyzed on sucrose gradients. Top panel, (——) A260 profile of the sucrose gradient; (Δ--Δ) ug of DNA per fraction. The bottom panel shows a 12% SDS polyacrylamide gel of the proteins in successive fractions across the sucrose gradient.

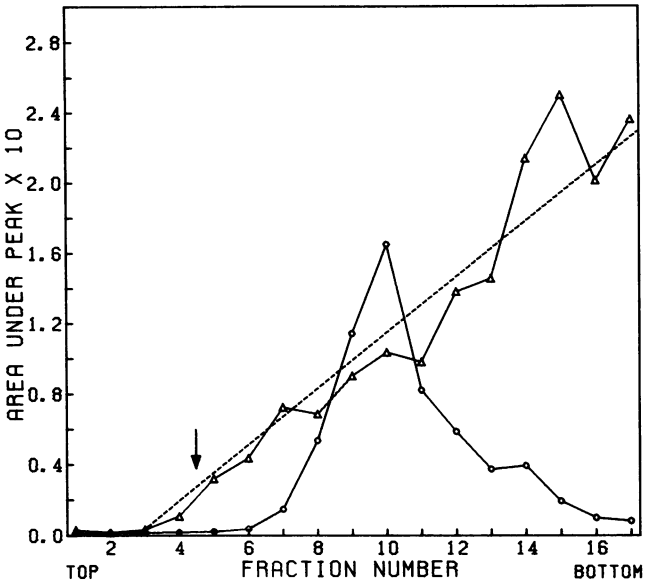

Figure 9. Distribution of the core histones H2a, H2b, H3, and H4 (Δ—Δ) and of the major 40S hnRNP proteins A1 and A2 (o—o) in the sucrose gradient shown in figure 8. The arrow marks the position of monomer nucleosomes in the gradient. The calculated linear regression for the histone data is shown by the dashed line. This data has a correlation coefficient of 0.966.

digest. This type of careful analysis of the nuclease released material fails to provide evidence for the non-random cleavage of HeLa chromatin as determined by the subsequent preferential release of polynucleosome structures of specific sizes.

DISCUSSION

In this report we have shown that hnRNP particles are released from HeLa nuclei along with monomer and polynucleosomes following brief digestion with micrococcal nuclease. The nuclease released hnRNP particles were identical to 40S hnRNP isolated by both low salt extraction and sonication procedures.

One surprising result of these studies was the close similarity between hnRNP isolated by the low salt extraction procedure and those isolated by sonication. Other investigators

have reported that the sonication procedure yields an hnRNP preparation with heterodisperse sedimentation properties and a more heterogeneous protein composition than the 40S hnRNP isolated by low salt extraction (8,9,23). Both procedures yield RNA-protein complexes which migrate at 40S in sucrose gradients. The protein components of these 40S complexes were essentially identical on one dimensional SDS gels with more than 65% of the total protein mass being the six protein bands with molecular weights of 32,000 to 44,000, previously identified as core hnRNP proteins (4,5). The one significant difference in the two preparations was the size of their RNA components, with the RNA of the sonicated particles being of higher molecular weight and considerably less degraded than the RNA of the STM extracted particles. It is possible that endogeneous nucleases are responsible for the release of monomer particles during our sonication procedure; however, this seems unlikely since lengthy extractions at room temperature and at 37°C were required for the release of a comparable quantity of particles by endogeneous nucleases during the low-salt STM extraction procedure.

The results of these studies further substantiate our contention that the 40S hnRNP particle exists as a discrete structural unit in some larger repeating complex *in vivo*. It is not obligatory that each 40S particle be exactly identical, and in fact it seems most likely that the monomer population would be somewhat heterogeneous based on our present understanding of the processing events which convert nascent hnRNA molecules to functional messenger RNAs. Monomer particles could serve to package the nascent hnRNA and at the same time act as the matrix on which processing occurs. This would lead to particle heterogeneity in both the size of the RNA component and in the associated accessory proteins or processing enzymes, and could even lead to variations in the ratios of core hnRNP proteins if one or more of these proteins serves primarily to mask the nascent RNA, protecting it from random nuclease attack.

While it was not our purpose to study higher order chromatin structure, we feel that the results presented in this paper have important implications for other studies using micrococcal nuclease as probe. A substantial portion of the nuclease

released A_{260} material migrating with polynucleosomes 6N to 9N in length in our preparations was shown to be RNA. When we looked for the preferential release of specific size classes of polynucleosomes by determining the distribution of DNA and of core histone molecules in our sucrose gradients, we found none. In figure 9 there is a peak of DNA and histones which could correspond to 16N polynucleosomes, but the magnitude of this peak is not sufficient to support a claim that it represents preferential release. Careful consideration of the expected sedimentation properties of polynucleosomes provide a more feasible explanation of this peak. Adding an additional nucleosome to a monomer or dimer structure will alter its hydrodynamic properties sufficiently to allow separation of the monomers, dimers, and trimers in sucrose gradients. However, adding an additional nucleosome to a 9N or larger polynucleosome structure will have little effect on its hydrodynamic properties, changing its molecular weight by only 10% or less and having little effect on its overall size and shape. As a result one would not expect to be able to separate larger polynucleosomes on sucrose gradients. This is supported by our experimental observation that polynucleosomes larger than 8N were never resolved on our gradients. We feel that the peak in the very brief digestion experiment is a mixed population of polynucleosomes 9N and larger which sediment to the lower region of the gradient.

Finally we suggest that the presence of RNA-protein complexes in eukaryotic nuclei will interfere with not only the types of studies we have described here, but also with studies of the rate of chromatin digestion with micrococcal nuclease as well as ultrastructural studies of chromatin organization. Care must be taken to differentiate between RNA and DNA when interpretating the results of studies utilizing micrococcal nuclease as an experimental probe.

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