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Preservation of Specific RNA Distribution within the Chromatin-depleted Nuclear Substructure Demonstrated by In Situ Hybridization Coupled with Biochemical Fractionation

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Abstract. Biochemical fractionation procedures previously shown to remove 95% of cellular protein, DNA, and phospholipid, were combined with fluorescence in situ hybridization to provide a critical evaluation of the retention and spatial preservation of specific primary transcripts within the chromatin-depleted nuclear substructure, operationally defined as the nuclear "matrix." This unique approach made it possible to directly address whether nuclear extraction procedures preserve, create, or destroy ribonucleoprotein filament structures. Comparison of nuclei before and after fractionation demonstrated that localized foci or "tracks" of specific nRNA are unambiguously retained in the nuclear matrix preparation. Two well-characterized nuclear fractionation procedures were used and three Epstein-Barr virus–infected cell types investigated, including latently and permissively infected cells carrying integrated or episomal genomes. The EBV primary transcripts as well as nucleolar RNA were preserved within the remaining nuclear substructure with unambiguous spatial and quantitative fidelity. Image processing and quantitative microfluorimetry, together with [^{3}H]thymidine labeling of DNA, show that essentially 100% of the RNA signal intensity remained after removal of 85% of the DNA. That the native RNA distribution was unchanged was shown in other experiments in which the same individual nRNA tracks were examined before and after fractionation. Results conclusively demonstrate that the tight restriction of RNA to highly localized sites is independent of bulk DNA removal and of extensive extraction of proteins and phospholipids. Hence, this work provides direct visual evidence that the primary transcripts studied are localized via their binding to, or comprising part of, nonchromatin nuclear substructure.

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COMPARED with the detailed biochemical and molecular information available on messenger RNA metabolism, relatively little is known as to how the complex systems responsible for the transport and processing of nuclear RNAs are integrated into nuclear structure. The fundamental question of whether mRNA precursors are synthesized in specific regions of the nucleus is unresolved and very little is known as to how transcripts destined for the cytoplasm are selectively transported from their site of synthesis to the nuclear pore. Relevant to these questions is the fact that the nucleus is an extremely viscous structure due to the enormous density of DNA, RNA, and protein that it contains. Hence it has been proposed that RNA is unlikely to be freely diffusing within this dense structure, but is more likely actively transported along a "solid-state" nuclear architecture (see for example Agutter, 1985) and/or through channels in chromatin possibly connected to nuclear pores (Blobel, 1985).

During the past 15 years a sizeable body of literature has provided evidence for a nonchromatin nuclear substructure, termed the nuclear matrix or scaffold (reviewed in Nelson et al., 1986; Schroder et al., 1987; Verheijen et al., 1988). This entity is operationally defined as the fibrillogranular material, visible through the electron microscope, which remains within the nuclear interior after fractionation with detergent to remove soluble material, salt extraction of insoluble proteins, and extensive DNase digestion to remove chromatin. Nucleolar bodies and proteins of the peripheral nuclear lamina (and pore complexes) are also retained in these preparations, and studies using agarose embedment to preserve nuclear structure have indicated that an intermediate filament-like protein fiber may be an underlying component of the internal matrix (Jackson and Cook, 1988). Most importantly, numerous studies from various labs (Faiferman and Pogo, 1975; Long et al., 1979; Berezney, 1980; Ciejeck et al., 1982; Jackson et al., 1981; Gallinaro et al., 1983; Ross et al., 1982; Mariman et al., 1982; Van Eekelen et al., 1981; Fey et al., 1986*a*), using increasingly refined matrix preparation methods, have shown that >90% of newly synthesized hnRNA is retained with the matrix and, in fact, may be an integral component of it necessary for maintaining nuclear structure (Fey et al., 1986*a*). The matrix has been im-

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plicated in hnRNA processing as well (Zeitlin et al., 1987). Other studies have suggested that active genes and replicating DNA are physically associated with the matrix (Ciejek et al., 1983; Jackson et al., 1981; Berezney, 1980) and that specific DNA sequences termed "MARs" (matrix attachment regions) bind to it with high affinity (see for example, Mirkovitch et al., 1984). All of these observations indicate that there exists a nonchromatin nuclear substructure that may play a significant role in such fundamental processes as chromatin organization, transcription, RNA processing and transport, and DNA replication.

Despite an extremely large body of evidence accumulated since the chromatin-depleted nuclear matrix was first described (Berezney and Coffey, 1974), its existence in vivo and its specific association with nuclear RNA is not fully accepted. The concept of the matrix as a major structural component of the nuclear interior, with broad functional implications, has received little recognition as part of mainstream cell and molecular biology (see for example, DeRobertis and DeRobertis, 1987; Alberts, 1989; Darnell et al., 1990; Lewin, 1990). While there may be several reasons for this hesitancy, a primary concern is that matrix structures visualized after nuclear fractionation may not accurately reflect structures that exist within intact cells, but could be an artifact of harsh preparation procedures, which trap or nonspecifically bind nuclear RNA. Because of the difficulty of investigating ultrastructure in extremely dense unfractionated nuclei, it is not possible to demonstrate that the RNP-containing fibrillogranular structures visualized by resinless section electron microscopy in extracted nuclei also exist within nuclei of intact cells. This limitation has constituted a major obstacle to directly demonstrating bona fide in vivo counterparts of matrix structures. The precise localization of specific nuclear RNAs in both fractionated and unfractionated nuclei could contribute significantly to addressing a basic controversy which has long pervaded this field.

The work described here investigates the interaction of nuclear RNA with the nonchromatin nuclear substructure using a different approach than previous work. The experimental strategy was to couple recently described high resolution in situ hybridization methodology (Lawrence et al., 1988, 1989) with nuclear fractionation procedures in order to visualize the distribution of a specific nuclear RNA both before and after nuclear extractions which remove the bulk of DNA, protein, and phospholipid. Our laboratory has previously reported direct visualization of specific primary transcripts within nonfractionated nuclei using fluorescence in situ hybridization (Lawrence et al., 1989). This work revealed a striking localization of specific viral transcripts within nuclei of lymphoma cells latently infected with Epstein-Barr virus (EBV). The fact that the transcripts were tightly localized to well-defined "tracks" or "foci" suggested that they are not free to diffuse, either because of spatial constraints or, perhaps, because they are physically bound to some nuclear structure. This model system provides an excellent opportunity to investigate whether a specific RNA can be visualized within nuclear matrix preparations. The objectives were (*a*) to address a key point as to whether this RNA is localized due to spatial constraints between masses of chromatin or via association with nonchromatin nuclear substructure, and (*b*) to help resolve an important issue concerning the extent to which these fractionation procedures preserve structures which exist within intact cells, particularly the ribonucleoprotein filaments.

For three different virally infected cell types, two different nuclear matrix preparation procedures were evaluated which previously have been thoroughly characterized and reported to preserve hnRNA (Fey et al., 1986*a*; Gallinaro et al., 1983). The primary procedure used (Fey et al., 1986*a*) has been very well characterized by electron microscopy, two-dimensional gel electrophoresis, and assays of total RNA, DNA, and protein retention. This fractionation procedure has been shown to remove >95% of phospholipid, 94% of DNA, and 95% of cellular protein, while retaining 76% of nuclear RNA (Fey et al., 1986*b*).

Materials and Methods

Cell Culture, Nuclear Preparation, and Fractionation

The Namalwa, B598, and BL2-B598 cells were grown in RPMI medium with 10% fetal calf serum (Gibco, Grand Island, NY) at 37°C. Cells were pelleted at 650 *g* for 5 min, resuspended in PBS, and cytospun onto glass microscope slides at a density of 2×10^5 cells/slide. Slides were air dried 5 min, then fixed in 4% paraformaldehyde in PBS for 5 min and stored in 70% EtOH at 4°C. Alternatively, cells were put through a series of extraction and digestion procedures before fixation in paraformaldehyde and stored, as described below.

For most experiments, cells were cytospun onto slides and fractionated according to Fey et al. (1986*a*), alternatively cells were fractionated first and then placed on slides. Cells were washed with PBS at 4°C and incubated in cytoskeleton (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM pH 6.8 Pipes, 3 mM MgCl2, 10 μM leupeptin, 2 mM vanadyl adenosine, and 0.5% Triton X-100) at 4°C for 10 min. Slides were then moved to an extraction buffer (250 mM ammonium sulphate, 300 mM sucrose, 10 mM pH 6.8 Pipes, 3 mM MgCl2, 10 μM leupeptin, 2 mM vanadyl adenosine, and 0.5% Triton X-100) for 5 min at 4°C. The chromatin fraction was removed from the remaining structure after digestion for 20–60 min at 20°C in a buffer identical to the CSK buffer but containing only 50 mM NaCl and 100 μg/ml bovine pancreas DNase I (Worthington Biochemical Corp., Freehold, NJ). DNase I digestion was terminated by putting slides into CSK buffer containing 0.25 M ammonium sulphate. The slides were fixed in 4% paraformaldehyde as described above. Previous studies showed that this procedure removed 94% of the DNA, >95% of the histones and 70% of the nonhistone nuclear proteins (Fey et al., 1986*b*).

The alternative fractionation procedure utilized was that described by Gallinaro et al. (1983). Cytospun slides were incubated in reticulocyte standard buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) in the presence of 10 μM leupeptin for 10 min at 0°C. Samples were then washed in 10 mM Tris-HCl pH 7.4, 100 mM KCl, 1.5 mM $MgCl_2$, 0.25 M sucrose, 10 μM leupeptin. Washed slides were digested by 100 μg/ml RNase-free DNase I for 60 min at room temperature. After digestion, samples were washed in 10 mM Tris-HCl pH 7.4, 400 mM KCl, 0.2 mM $MgCl_2$, 0.25 M sucrose, 10 μM leupeptin.

For analysis of interphase nuclei and metaphase chromosomes, standard cytogenetic preparation were used, as previously described (Lawrence et al., 1988, 1989). Cells were incubated at 37°C with 0.015 μg/ml of colcemid (demicolcine) for 2–3 h, pelleted and resuspended in 0.075 M KCl at 37°C for 17 min. The cell suspension was fixed in three changes of fresh 3:1 methanol/acetic acid and gently dropped onto EtOH-cleaned slides in a humid environment to promote spreading. Slides were air dried overnight and stored at −80°C with desiccant.

Probes

The EBV probes were provided by James Skare (Skare and Strominger, 1980) and represent the Bam V fragments of the genome. DNA was nick translated by established procedure using biotinated-7-dATP (Enzo Diagnostics, Inc., New York). Probes were labeled with biotin and sized on agarose gels to assure fragment sizes were in the range of 200–500 nucleotides (Lawrence and Singer, 1985; Singer et al., 1986; Lawrence et al., 1989).

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Hybridization

Briefly, hybridization and detection was as previously described (Lawrence et al., 1988, 1989). Nuclear preparations were rinsed for 10 min in 2× SSC, and dehydrated through cold 70 and 95% EtOH for 5 min each before air drying. For each sample, 50 ng of probe, 10 ng of sonicated salmon sperm DNA and 10 ng of *Escherichia coli* tRNA were suspended in 10 μl deionized formamide and heated at 70°C for 10 min. The final hybridization buffer consisting of 50% formamide, 20% dextran, 10% BSA, and 4× SSC was added and then the slides were incubated at 37°C overnight.

Detection and Microscopy

Samples were incubated in 2 μg/ml fluorescein-avidin in 4× SSC, 1% BSA for 30 min at room temperature. Samples were then rinsed at room temperature for 10 min each in 4× SSC, 4× SSC with 0.1% Triton, and then 4× SSC. For visualization of total DNA, samples were stained with propidium iodide for 1 min at 0.5 μg/ml in PBS, or DAPI for 20 min in PBS, or acridine orange for 20 min. Samples were visualized at 1,000× using a Zeiss Axiophot microscope equipped with epifluorescence filters. Color photographs were taken on Ektachrome 400 with exposure times of 1.5–2 min for propidium or fluorescein and 5–20 s for DAPI.

Microfluorimetry

A CCD camera interfaced with a digital imaging microscope was used to record fluorescent images. The intensity of the DAPI and the fluorescein signals were determined by computer analysis of the images. Nuclei were chosen randomly for the intensity measurement by using a uniform box size for DAPI and the varied box for signals. Measurements of nonspecific background fluorescence were performed in the area free of cells using a box size similar to that for DAPI.

Results

The in situ hybridization procedure has been previously described (Lawrence et al., 1988, 1989) and used DNA probes nick translated with biotin and detected with fluorescein-avidin using epifluorescence microscopy. The study used primarily Namalwa human lymphoma cells latently infected with EBV and carrying two copies of the viral genome very closely integrated on one homolog of chromosome 1 (Henderson et al., 1983; Lawrence et al., 1988). Nuclear RNA distribution was also investigated in B958 cells productively infected with ~50 episomal viral genomes and in a second latently infected line (BL2-B958) carrying a single integrated viral genome. The primary probe utilized was the Bam HI W fragment of the EBV genome (Bam W) (Skare and Strominger, 1980; described in Lawrence et al., 1989), which previous experiments have shown provides a good test system for the detection of EBV nuclear RNAs due to its size and relative abundance within the nucleus. The Bam W sequences comprise up to 20 kb in the primary transcript, but are extensively spliced to represent only 0.9 kb of the mature EBNA mRNA, present in very low levels in cytoplasmic poly(A) RNA (Van Santen et al., 1983; Dambaugh et al., 1986).

nRNA Distribution within Intact Paraformaldehyde-fixed Cells Compared with Nuclei of Cytogenetic Preparations

Before considering the effect of nuclear fractionation procedures, which use paraformaldehyde fixation, it is instructive to compare the distribution of nuclear RNA in intact, paraformaldehyde-fixed Namalwa cells with nuclei of cytogenetic preparations, upon which much of our previously reported work was based (Lawrence et al., 1989). Nuclei within these preparations have been swollen in hypotonic solution, fixed in methanol/acetic acid, and placed onto glass slides, with the result that the nuclear diameter is expanded to ~1.5–2 times that of paraformaldehyde-fixed intact cells. In both types of preparations the Bam W transcripts are tightly restricted to a single site (occasionally two sites), the accumulated transcripts forming a clearly defined focus or track (Fig. 1, *A–C*). Previous analysis demonstrated that these tracks extend from the nuclear interior where the viral genome is localized (Lawrence et al., 1989). While the nRNA formation tends to be elongated in both types of preparations, the track is consistently more linear and often dramatically elongated in the swollen nuclei (compare Fig. 1, *A* and *B* with *C*). This comparison provides an initial insight into the relationship between nRNA distribution and nuclear structure, in that the more elongated nature of the tracks within swollen nuclei suggests that the RNA may be attached to, or comprise, a nuclear structure that becomes distended during osmotic swelling. If the primary transcripts were free to diffuse, it would be expected that the cytogenetic preparation would result in less, rather than more, pronounced localization. Also consistent with the tight binding of this nRNA within the nucleus is the observation that nuclear transcripts are stably retained after methanol/acetic acid fixation and hybridization, conditions previously shown to result in loss of 80–90% of cytoplasmic mRNAs (Lawrence and Singer, 1985).

Effects of Detergent Extraction on nRNA Distribution

As shown in Fig. 1 *C*, nuclei of unextracted paraformaldehyde-fixed cells stained for total DNA with propidium iodide or DAPI show a solid, evenly dense appearance with a well-defined nuclear border. The EBV Bam W RNA hybridization signal is observed in >90% of nuclei, with the accumulated RNAs forming either linear, twisted, or focal structures, and occasionally the signals were branched. The possibility that the signals represent the detection of the EBV genome can be excluded because: (*a*) the nuclear DNA in these experiments was not denatured and, therefore, does not hybridize under the conditions used (Lawrence et al., 1989, 1990); (*b*) after digestion with RNase, hybridization signals were completely absent in nondenatured samples (results not shown); (*c*) previous work showed signals were removed by actinomycin D and are specific to transcribed EBV sequences (Lawrence et al., 1989).

The first step in nuclear fractionation was to treat cells with Triton X-100 as specified in Materials and Methods, thereby permeabilizing the cell membranes and releasing soluble cellular components. As previously described (Fey et al., 1984, 1986*a*) this extraction removes phospholipids and ~70% of soluble cell protein but does not influence the DNA content. Light microscopic observations showed that the pattern of propidium and DAPI staining of the extracted nuclei was not noticeably different from that of the intact cell (Fig. 1 *D*). In situ hybridization demonstrated that the EBV nuclear transcripts were detected after Triton extraction in almost all nuclei. Further, the overall appearance and intensity of the nuclear RNA tracks within the extracted nuclei (Fig. 1 *D*) were identical to those of the intact cell nuclei (Fig. 1 *C*). Hence Triton extraction does not alter the retention or localization of this nRNA.

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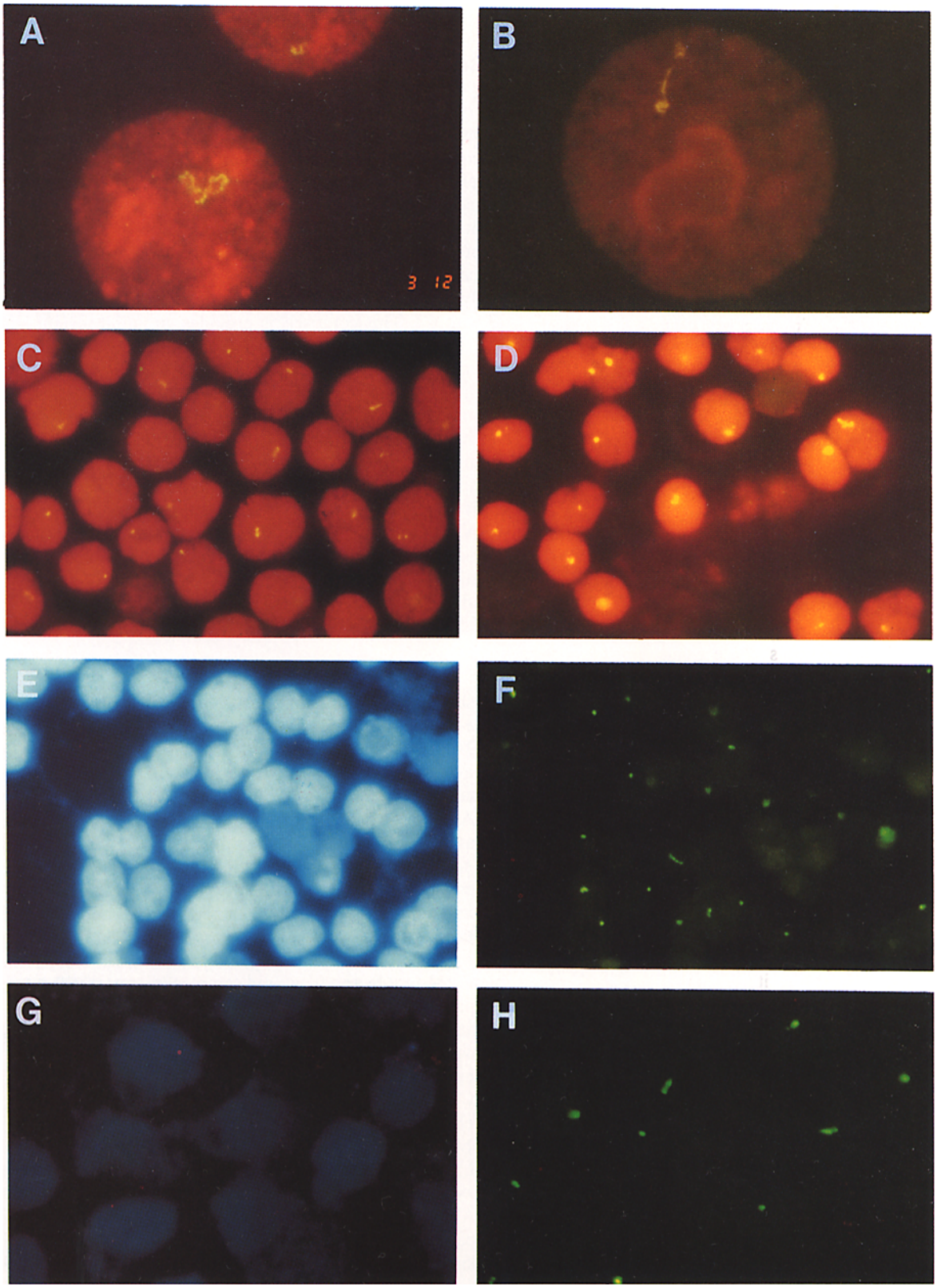


Figure 1. Fluorescent detection of nuclear RNA within cytogenetic preparations, intact cell and nuclear matrix preparations. Biotinated probes for EBV Bam W RNA were hybridized in situ to latently infected Namalwa cells (not denatured) and specific hybridization detected with fluorescein-avidin (*yellow*). Nuclei were stained with either propidium iodide (*red*) or DAPI (*blue*). *A* and *B* show hybridization to cytogenetic preparations of Namalwa cell nuclei, in which the RNA tracks have a particularly elongated configuration. (*C*) Tracks of EBV Bam W RNA in nuclei of paraformaldehyde-fixed intact Namalwa cells. Exposure, 1.5 min. (*D*) Hybridization to Bam W RNA in nuclei of cells extracted with Triton X-100 for 5 min. Exposure, 2 min. (*E*) DAPI fluorescence staining of Triton-extracted Namalwa cell before fractionation gives extremely bright fluorescence intensity of total nuclear DNA. Exposure, 20 s. (*F*) Fluorescein-avidin detection of Bam

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RNA Localization in Nuclei Fractionated with Detergent, DNase, and Ammonium Sulphate Extraction

A much more extensive fractionation of the nucleus was then undertaken after detergent extraction. After Triton treatment, chromatin was digested with RNase free DNase I and eluted with ammonium sulphate in the presence of RNAse inhibitors (Fey et al., 1986*a*). Chromatin is cut principally between nucleosomes and the digested DNA and associated proteins eluted by ammonium sulphate. In addition to removing most of the DNA and phospholipid, this procedure removes >95% of histones and 70% of nonhistone nuclear proteins. Several studies using one- and two-dimensional gels have shown the remaining nuclear fraction to contain a specific but heterogeneous set of nonhistone proteins (reviewed in Nelson et al., 1986; Schroder et al., 1987; Verheijen et al., 1988).

As illustrated in Fig. 1, *E* and *G*, the intensity of DAPI staining, which is directly proportional to DNA content (Coleman et al., 1981), is greatly diminished in the fractionated nuclei. The very pale residual DNA stain is evenly distributed throughout the nuclei. With propidium iodide, which stains both DNA and double-stranded RNA, the interior of the nucleus appeared devoid of staining after fractionation, with about one to four nucleolar bodies remaining in each nucleus (compare Fig. 1, *C* and *D* with Fig. 2 *A*). Based primarily on morphology, the large dense structures observed in matrix preparations by electron microscopy are considered to be nucleoli (Fey et al., 1986*a*). That these dense bodies are nucleoli was confirmed in our experiments by several observations. (*a*) While consistently observed after propidium iodide staining of both DNA and double-stranded RNA (Barni and Gerzeli, 1985), fully extracted nuclei stained for just DNA with DAPI exhibit uniform dim fluorescence (Fig. 1 *G*). (*b*) RNase H–digested samples stained with propidium iodide had a clean, empty nuclear interior with no nucleolar bodies. (*c*) Acridine orange staining of total RNA in intact cells showed bright staining nucleoli of similar size, shape, and number (not shown). Hence, in addition to providing evidence that these bodies are nucleoli, these results demonstrate that the ribosomal RNA itself is well preserved at its expected site within the preparation.

In situ hybridization was then applied to determine how removal of the bulk of the nuclear contents, including lipids, soluble and insoluble proteins, and DNA, would affect the retention and, moreover, the distribution of a specific mRNA precursor. Results were unambiguous, with the nRNA tracks still clearly observed in the "empty" nuclei (Fig. 1, *E–H* and Fig. 2, *A* and *B*). One or two tracks or foci were present in almost every nucleus, as in intact or Triton-treated nuclei. The formations of hnRNA were structurally indistinguishable from those in unfractionated nuclei. In addition, the fluorescence intensity of the hnRNA tracks was essentially the same as in nuclei before digestion (see below). These experiments were repeated numerous times with reproducible, consistent results. Fractionation was generally performed with cells on glass slides, but similar results were obtained with cells extracted in suspension. To assure that DNase conditions were optimal for chromatin removal, the effects of DNase concentration and digestion time were examined. DNase I was used in two concentrations (0.1 or 1 mg/ml) for 20, 40, or 60 min. In all samples, the general appearance and density of the nucleus was similar and the fluorescence hybridization signals identical (not shown). This indicated that the DNase 1 digestion reaction was rapid and essentially complete in 20 min, hence the 60-min digestion used in most experiments was more than sufficient for removal of bulk DNA (see below). In contrast, when cells were treated with RNase A before hybridization as a negative control, the hybridization signals were completely absent (not shown).

To address whether these results could be reproduced with a different matrix isolation procedure, a second method was used with 0.4 M KCl elution (Gallinaro et al., 1983) instead of ammonium sulphate (Materials and Methods). In general very good retention of nRNA tracks and foci were observed using this procedure (Fig. 2 *B*), although, in two of five experiments there was a slight diminution of the hybridization signals. Hence retention of the RNA within the matrix fraction is not unique to the ammonium sulphate protocol, but may provide a good assay for how well a given protocol or a given experiment has preserved native RNP structures.

Quantitation of DNA and RNA Retention

Results of the visual analysis described above were clear cut, in that RNA hybridization signals were not noticeably less intense after fractionation while nuclear DNA staining was greatly reduced. Removal of nuclear DNA is a critical and uniformly accepted criterion for preparation of the nuclear residue or "matrix." To provide objective confirmation and quantitation of these results, DNA loss was measured by [^{3}H]thymidine labeling and specific RNA hybridization signals were quantitated using microfluorimetry in large numbers of individual cells before and after fractionation. As shown in Fig. 1, there was extremely little intercell variation within an experiment. Quantitative results are summarized in Fig. 3 for the most frequently used fractionation procedure (Fey et al., 1986*a*). For two experiments with a total of 12 samples, an average of ~85% of radioactively labeled DNA was removed by the digestion procedures described above. This result was confirmed by a different strategy, using cells that had been fully extracted in suspension and then cytospun onto slides, in which 86% of DNA was removed.

Quantitation of specific hybridization signals was made possible by the use of quantitative microfluorimetry using digital imaging microscopy. The intensity of fluorescent signals after hybridization to the viral RNA was quantitated in over 50 randomly selected individual nuclei in both fractionated and unfractionated preparations. The hnRNA tracks in the cells before and after DNase treatment were virtually

W RNA in the same nuclei shown in *E*. Exposure, 1.5 min. (*G*) Same as *E* except that cells were fully extracted with DNase and ammonium sulphate (Materials and Methods). Exposure times were identical to *E*. Loss of DAPI fluorescence indicates loss of total DNA. (*H*) Detection of Bam W RNA in the nuclei shown in *G*. Note the great decrease of DAPI intensity in the DNased nuclei and the indistinguishable fluorescence intensity and morphological characteristics of the specific RNA signals in fractionated and unfractionated nuclei.

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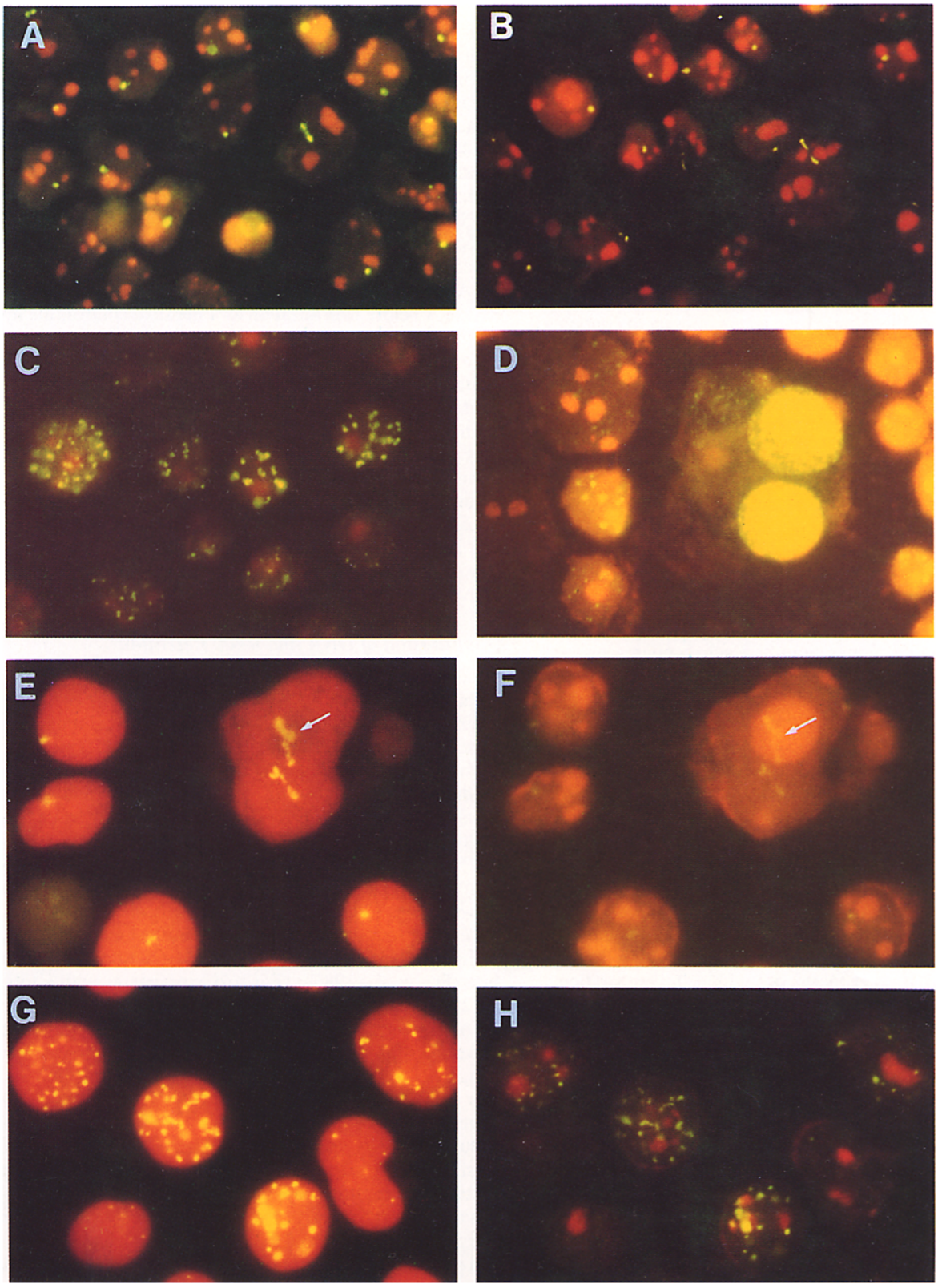


Figure 2. Fluorescence localization of nuclear RNA within B95-8 and Namalwa cells, before and after extraction. Nuclei were stained with propidium iodide (*red*). All fractionations used DNase and the ammonium sulphate procedure (see Materials and Methods except where otherwise noted). Exposure, 2 min. (*A*) Tracks of Bam W RNA (*yellow*) in nuclei of extracted Namalwa cells. Nucleoli remain in the matrix fraction and are stained by propidium iodide. (*B*) Same as *A* except that DNase digested nuclei were eluted using the 0.4 M KCl procedure (see Materials and Methods). (*C*) B958 cells extracted as in *A*. These cells carry numerous episomal genomes and exhibit many viral RNA foci or tracks, all of which are retained with the nuclear matrix fraction. (*D*) B958 cells, extracted as in *A*, illustrating a small fraction of highly productive cells with strong hybridization throughout the nuclei and some cytoplasmic signal. (*E* and *F*) The

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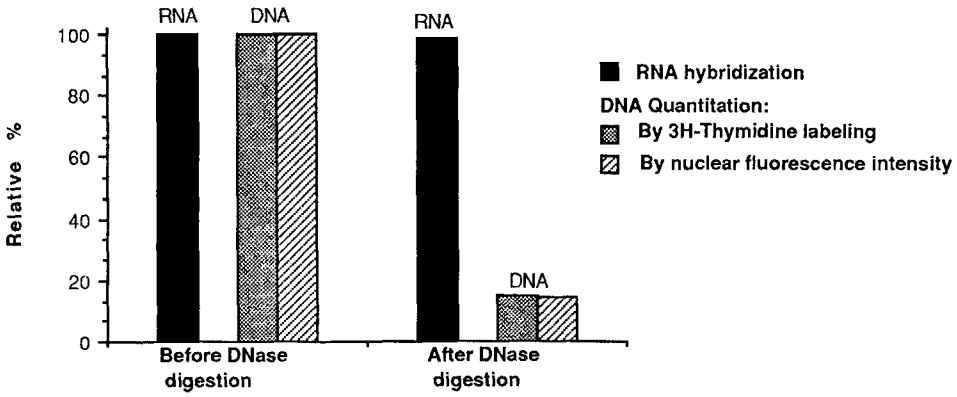


Figure 3. Quantitation of nRNA and DNA retention in nuclei of Namalwa cells. For quantitation of DNA removed by fractionation, cells were labeled for 24 h with 10 μCi/ml [^{3}H]thymidine and counted in a scintillation counter. Eight slides of each group with or without DNase I digestion were measured from two experiments. For quantitation of RNA hybridization signal and DNA intensity with DAPI, image processing was used. The density of either fluorescent hybridization signal or nuclear staining was measured by microfluorimetry in 50 randomly selected nuclei for each group.

identical in their average fluorescence intensity (Fig. 3). Remarkably, the close to 100% retention of the specific nRNA was higher than the 76% retention reported for total hnRNA by this procedure (Fey et al., 1986*a*).

Localization and Retention of Viral nRNA in Other Permissively and Latently Infected Cell Types

To address whether the localization of this viral RNA and its stability and retention throughout these procedures is specific to the cell line studied, two other EBV-infected cell lines were analyzed. A second latently infected line, BL2-B958 with a single integrated viral genome exhibited a distribution of nuclear transcripts indistinguishable from Namalwa cells and a similar retention of nRNA after fractionation (not shown). More importantly, Fig. 2 *C* (see also Fig. 2, *G* and *H*) illustrates results of hybridization to permissively infected B958 cells that carry numerous episomal viral genomes. The hybridization pattern is very different from Namalwa cells, with many well-defined tracks of Bam W nRNA throughout the nucleus. Hence, each of numerous active episomal genomes apparently produces an nRNA track. Nuclear fractionation, as described above, demonstrates that these foci of RNA are completely retained after nuclear fractionation. It can be concluded, therefore, that the primary transcripts from nonintegrated DNA are also physically bound within nuclear substructure.

The vast majority of B958 cells had many sites of localized transcripts within their nuclei, as illustrated in Fig. 2 *C*, however a small fraction (~3–5%) had extremely bright fluorescence with the highest concentrations more evenly distributed throughout the nucleus (Fig. 2 *D*). This is consistent with the frequency of cells in this line that enter a highly productive state. It was of interest, therefore, that the majority of RNA produced in these cells was also retained within the nuclear matrix fraction (see Fig. 2 *D*). Hence, the results observed are not unique to the Nawalwa cell line or to latently infected cells, but are a common property of different cell lines and cells in different states of expression of this nRNA.

Visualization of nRNA in the Same Cells before and after Fractionation

The above results strongly indicate that the RNA studied is quantitatively retained within fractionated nuclei, with no apparent change in morphology or distribution of the specific RNA tracks. An especially convincing demonstration that native ribonucleoprotein filament structures remain unperturbed in the chromatin-depleted nucleus would be to visualize these structures in the same cell both before and after fractionation. This would further address whether the track of RNA could be maintained within the matrix preparation by virtue of attachment at one end, for instance, to the nuclear lamina or pore. Experiments were designed to address whether the RNA formation shifts position during fractionation.

Hybridization was performed and fields of cells photographed and marked. Samples were then fractionated by Triton, DNase digestion, and salt elution as described above. The same cells were then relocated and photographed to compare with the RNA distribution before fractionation. As illustrated for Namalwa cells in Fig. 2, *E* and *F* and B958 cells in Fig. 2, *G* and *H*, the nuclear staining with propidium iodide indicates that the fractionation procedure, which includes an extensive DNase digestion, was still very effective in removing nuclear DNA despite the prior brief fixation and hybridization. Results show clearly that for both cell types the RNA signals are not only still present, but are essentially identical in appearance and position. Neither end of the

same Namalwa cell nuclei before (*E*) and after (*F*) extraction. Note the identical position of the nRNA tracks after extraction. It is important to note that the fluorescein hybridization signal is diminished only due to bleaching during prephotography as demonstrated by quantitation of nRNA and DNA retention (Fig. 3). The propidium iodide staining, in contrast, does not fade during photography due to different properties of this dye. (*G* and *H*) The same nRNA tracks in nuclei of B958 cells before (*G*) and after (*H*) fractionation. Again note any diminution of fluorescein (*yellow*) signals is due to fading during photography. Nuclear staining indicates that the DNase digestion conditions used were effective after fixation and hybridization.

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RNA tracks show any displacement after DNase digestion, further evidence that they are held by or form substructures within the chromatin-depleted nucleus. It is important to note that the decreased intensity of the fluorescein signals after fractionation is due to the fading that occurs when the slide is exposed to light for the first photograph (before fractionation). On the same slide, hybridization signals in areas that were not exposed to light showed similar intensity before and after digestion, as documented by the quantitative studies described above. It was a consistent and striking observation that, by procedures that might be expected to be harsh or disrupting, and that systematically remove the bulk of the nuclear contents, these formations of specific nRNA show no indication of movement or loss.

Discussion

This work provides direct visual evidence supporting the retention of a specific nuclear RNA within the chromatin-depleted nuclear substructure. The novel approach used allows not just quantitation of the RNA, but a qualitative evaluation of its spatial distribution before and after nuclear fractionation. Nuclear matrix literature frequently acknowledges the difficulty of unequivocally demonstrating that the RNP-containing structures observed by electron microscopy are not created during nuclear extraction in low or high ionic strength salts (see for example Schroder et al., 1987; Agutter et al., 1985; Jackson and Cook, 1988; He et al., 1990). A direct critical test of this is made possible by the work described here. The RNA studied is unequivocally retained within the nonchromatin nuclear substructure, with no appreciable change in either the quantitative or qualitative appearance of the hnRNA track. Retention of nuclear RNA formations in fractionated nuclei was observed in three different virally infected cell types using two different matrix isolation procedures. Quantitative image processing and microfluorimetry was used to confirm that close to 100% of the EBV nRNA present within intact nuclei still remained after nuclear fractionation, whereas at least 85% of DNA was removed. These results demonstrate that native RNP distribution is preserved throughout these procedures and further indicate that the striking localization of the nRNA within the nucleus is a consequence of its binding or comprising internal structures which prevent free diffusion. Results of this coupling between high resolution hybridization and nuclear fractionation corroborates a body of work based on biochemical and ultrastructural studies supporting the existence of a nonchromatin nuclear structure with which hnRNAs are intimately associated.

Our previous results demonstrating a highly localized RNA track suggested that the nRNA was either associated with nuclear structure, or perhaps compressed into a narrow space or "channel" between masses of chromatin. Results presented here demonstrate that the bulk of the chromatin can be removed from the nucleus, and the nRNA maintains its position, hence the nRNA is not localized by spatial constraints confining it to a channel. Although it is possible that the nRNA track or focus is tethered at one point to the nuclear lamina (or pore) that remains in the matrix preparation, the nRNA tracks cannot be directly attached along their length to the lamina because previous work demonstrated that they extend into the nuclear interior where the viral genome is localized (Lawrence et al., 1989). This observation has been recently confirmed by confocal microscopy and three-dimensional analysis on suspension cells. (Bauman, J., and J. B. Lawrence, manuscript in preparation). The conclusion that the primary transcripts are "bound" to nuclear substructure is not intended to exclude the possibility that the RNPs actually comprise this substructure, as has been suggested previously (Fey et al., 1986*a*; Nickerson et al., 1989). It is possible that the nuclear RNAs studied constitute part of the matrix "core filaments" recently described (He et al., 1990), however further investigations would be required to establish this.

The nuclear RNA studied was transcribed from the EBV genome and codes for a low abundance cytoplasmic nRNA for EB nuclear antigens expressed in both latently and productively infected cells. Hence, in all three infected cell lines investigated the nRNA is processed and transported to the cytoplasm. However the target sequences represented in the mature nRNA are very small relative to the primary transcript (0.9 vs. 21 kb), diffusely distributed, and much less abundant (three molecules per cell; Dambaugh et al., 1986), and thus are less detectable in the cytoplasm. The localization of the primary transcripts and their retention within the matrix preparations are not properties peculiar to a given EBV-infected cell line, but were demonstrated in three different cell lines, including latently and productively infected cells. Interestingly, the B958 cell line carries numerous episomal genomes, each of which apparently produces an RNA track, all of which are in a bound state unperturbed by nuclear fractionation. Hence RNA produced by episomal as well as integrated genomes is physically associated with the nonchromatin nuclear substructure. It is interesting to speculate that the abundance of viral RNAs in the nucleus relative to the cytoplasm of nonproductively infected cells could reflect control of expression related to release from the nuclear matrix.

It cannot be assumed that the viral model system will be representative of primary cellular transcripts in general, however these results provide evidence that this specific RNA is localized by virtue of its association with substructure. Regardless of whether the term nuclear matrix is used to describe the remaining structure, the significant point is that the striking morphological localizations of this nRNA are independent of bulk DNA and extensive protein and lipid extraction. The fact that the RNA distribution is precisely preserved in a form indistinguishable from its in vivo distribution makes it unlikely that it is merely entrapped or aggregated within the nuclear fraction. If this were the case, we would expect that some quantitative or spatial alteration of its distribution would have been discernible. A general conclusion, independent of the model system used, is that carefully characterized nuclear fractionation procedures (Fey et al., 1986*a*; Gallinaro et al., 1983) can preserve, with a remarkable fidelity, the distribution of a specific primary transcript.

While some types of nuclear RNA may have primarily a structural role, the functional significance of pre-mRNA association with the matrix is in all likelihood directly related to transport or processing, or both. The rapid and selective exit of RNA from the nucleus almost certainly requires some form of vectorial transport, for which the matrix may provide the framework. There is an increasing awareness of cel-

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lular structure or compartmentalization in facilitating cell biochemical functions (for example the binding of enzymes involved in electron transport within the mitochondrial membrane). The possibility of a role for a nonchromatin substructure or matrix in RNA processing is made stronger by evidence that association of RNA processing components with the nuclear matrix fraction can significantly increase the speed of these reactions (Zeitlin et al., 1989). In keeping with the consideration that the RNPs themselves may comprise this substructure, it is possible that pre-mRNA molecules from a given gene somehow associate or interact with each other so as to promote an ongoing chain of transcription, processing, and transport. This is consistent with the observation that inhibition of transcription with actinomycin D appears to halt the movement of previously transcribed EBV RNAs toward the nuclear periphery (Lawrence et al., 1989).

The experimental approach demonstrated here, because of the advantage of allowing visualization of specific RNAs or DNAs before and after fractionation, should be broadly applicable to a host of questions concerning the interrelationship of nuclear structure with RNA transport and processing, and with chromatin organization. The ability to precisely localize single genes or their primary transcripts, as well as more abundant sequences, is in itself a powerful approach to studies of nuclear organization. The work presented here demonstrates the feasibility and value of wedding this approach with various biochemical fractionation procedures in order to unravel the underlying structural and organizational principles of the nucleus and chromosome.

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