The chicken lysozyme gene 5’ MAR and the Drosophila histone SAR are electroelutable from encapsulated and digested nuclei

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SUMMARY
Cultured chicken cells were encapsulated in agarose microbeads, lysed in a near-physiological buffer and resulting encapsulated nuclei were digested with a restriction enzyme and electroeluted. After removal of ~97% of the chromatin, the nuclear lamina, residual nucleoli and an internal nuclear network remained. The majority of nascent RNA was also recovered in digested and electroeluted nuclei. Surprisingly, however, the chicken lysozyme gene 5’ MAR was quantitatively electroeluted from digested nuclei of expressing and non-expressing cells, as well as the promoter region and the coding sequence. When encapsulated nuclei were digested partially, the proportion of elutable 5’ MAR chromatin was comparable to that of elutable bulk chromatin. Furthermore, after digestion of encapsulated nuclei from Drosophila Kc cells, the histone SAR was electroeluted to the same extent as bulk chromatin. We conclude that the lysozyme gene 5’ MAR and the histone SAR are not permanently attached to a nuclear matrix or scaffold.

Key words: Nuclear matrix, Scaffold, MAR, SAR

INTRODUCTION
Various biochemical techniques have cooperated to show that interphase chromatin is organized into looped domains. Early studies used histone-depleted chromatin of Drosophila interphase cells, treated it with the intercalating dye ethidium bromide, and followed the resulting structural changes by sedimentation (Benyajati and Worcel, 1976). It was concluded that Drosophila chromatin is organized into topologically separated domains of ~85 kb in size. In a second biochemical approach it was found that ‘active’ genes exhibit a general elevated DNase I sensitivity and that the regions of elevated sensitivity mostly include extended 5’- and 3’-flanking sequences containing cis-acting regulatory elements (Weintraub and Groudine, 1976). In individual cases, such as the domains of the chicken lysozyme gene, the human apolipoprotein B gene, and the human β-globin gene locus, it was shown that the DNase I-sensitive domain coincides with the functional gene locus, i.e. the coding region plus all cis-acting regulatory elements (Jantzen et al., 1986; Grosveld et al., 1987; Phi-Van and Strätling, 1988; Levy-Wilson and Fortier, 1989). Thus the idea emerged that domain boundaries are attached to a nuclear matrix and thereby generate the organization of chromatin into topologically separated regions.

Using a different technique, which extracts interphase nuclei with the detergent lithium diiodosalicylate (LIS) followed by digestion with restriction enzymes, specific DNA fragments (scaffold attached regions = SARs) partition with an insoluble scaffold fraction (Mirkovitch et al., 1984). For a few gene loci (the chicken lysozyme gene and the human apolipoprotein B gene domains) the LIS extraction method as well as the procedure, which uses incubation of defined fragments with nuclear matrices, have been applied. In these cases the sequences defined as MARs coincide with those defined as SARs (Phi-Van and Strätling, 1988; Levy-Wilson and Fortier, 1989).

Electron micrographs of histone-deprived metaphase chromosomes shown by Laemmli et al. revealed loops of DNA emanating from a central proteinaceous scaffold (Paulson and Laemmli, 1977; Marsden and Laemmli, 1979). Specific proteins, such as DNA topoisomerase II and the scaffolding protein Sc2, are components of the chromosomal scaffold, which is thought to be a functional equivalent to the nuclear matrix in interphase nuclei (Lewis and Laemmli, 1982; Boy de la Tour and Laemmli, 1988; Saitoh et al., 1994). Thus visualisation of loops merged with the evidence of chromatin domains into the concept of a loop-domain organization of chromatin.
Though the evidence of a proteinaceous chromosomal scaffold is fairly well documented, it is still uncertain whether chromatin loop-domains are generated by attachment of specific sequences to a rigid framework structure. To approach this question we selected a relatively mild technique that employs nuclei encapsulated in agarose microbeads and near-physiological buffer conditions (Jackson et al., 1988). Chromatin contained in such nuclei was digested with a frequently cutting restriction enzyme and then probed by electroelution. Using this technique Cook et al. previously demonstrated that the majority of chromatin is electroelutable from digested nuclei but nascent RNA and nuclear transcription domains are not (Jackson and Cook, 1985; Jackson et al., 1993). Furthermore, replicationally active territories are also retained in electroeluted nuclei (Hozák et al., 1993). Applying the technique to an ectopic copy of the fushi tarazu SAR in Drosophila embryo nuclei, it was shown that this SAR is electroelutable as readily as a non-SAR sequence (Eggett and Jack, 1991). It was concluded that the fushi tarazu SAR is not attached to a nuclear matrix.

The chicken lysozyme gene domain that we used here as a model system is schematically depicted in Fig. 1. In expressing cells, such as the tubular gland cells of the oviduct, the domain containing ~20 kb of DNA exhibits an ~2- to 3-fold elevated DNase I sensitivity (Jantzen et al., 1986). MARs, each domain containing ~20 kb of DNA exhibits an ~2- to 3-fold elevated DNase I sensitivity (Jantzen et al., 1986). Furthermore, replicationally active territories are also retained in electroeluted nuclei (Hozák et al., 1993). Applying the technique to an ectopic copy of the fushi tarazu SAR in Drosophila embryo nuclei, it was shown that this SAR is electroelutable as readily as a non-SAR sequence (Eggett and Jack, 1991). It was concluded that the fushi tarazu SAR is not attached to a nuclear matrix.

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MATERIALS AND METHODS

Cell culture
The chicken cell lines HD11 (Leutz et al., 1984) and DU249 (Langlois et al., 1974) were grown in 75 cm² tissue culture flasks or 10 cm diameter dishes (Greiner) in Iscove’s modified Dulbecco’s medium (with L-glutamine; Life Technologies, Inc.) supplemented with 8% fetal calf serum (Life Technologies, Inc.), 2% chicken serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO₂ (Ahne and Strätling, 1994). Drosophila Kc cells (Echalier and Ohanessian, 1970) were grown in 75 cm² tissue culture flasks or in 1 l spinner culture flasks in D-22 insect medium (Sigma) supplemented with 5% fetal calf serum (Boehringer, Mannheim), 100 units/ml penicillin, and 100 μg/ml streptomycin at room temperature. All cell lines used were free of mycoplasma contamination.

Cell encapsulation, cell lysis, chromatin digestion, electroelution and labeling procedures
Cells were encapsulated (3×10⁶/ml) in 0.5% agarose microbeads (Waitz and Loidl, 1988; Hempel et al., 1993), washed in ice-cold phosphate-buffered saline, and lysed on ice in ‘physiological’ buffer containing 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100 by three changes of lysis buffer (each 10 minutes) (Jackson et al., 1988). ‘Physiological’ buffer contains 130 mM KCl, 10 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM Na₂ATP, 1 mM dithiothreitol adjusted to pH 7.4 with 100 mM KH₂PO₄ (Jackson et al., 1988). Then beads were washed twice in ‘physiological’ buffer (each 10 minutes) and digested at 32°C with 2,500 units of HaeIII (45 minutes) or HinfI (90 minutes) per ml of a 50% bead suspension. Reactions were stopped by cooling on ice. Electroelution was performed in dialysis bags (Visking) at 2 V/cm for 5 hours in ‘physiological’ buffer. We next used a 30 cm × 26 cm elution device with a 12 cm broad cooling plate (cooled with circulating ice-water), on which the dialysis bags were placed (Hempel et al., 1993). Outlets to a circulating pump allowed rapid exchange with a reservoir containing 5 l of ‘physiological’ buffer. In order to prevent contact of the electrolytes with circulating buffer, the portions of the device, which harbored the electrodes, were separated from the elution chamber by 1% agarose plugs and contained 40 mM Tris-HCl, pH 8.3, 20 mM Na-acetate.

In labeling experiments encapsulated HD11 cells continued to grow overnight as a 25% suspension in culture medium containing [methyl-¹⁴C]thymidine (3.7-37 kBq/ml) (Amersham Buchler). On the following day, beads were washed and cells were pulse-labeled for three minutes at 37°C with [5.6-¹⁴]thymidine (3.7-37 kBq/ml) (Amersham Buchler). Labeling was stopped by the addition of 50 ml ice-cold phosphate-buffered saline. After four washes in phosphate-buffered saline, cells were lysed and washed as described, except that buffers were supplemented with 25 units/ml RNase inhibitor (Boehringer, Mannheim). Then samples were split in half. One half was subjected to limited chromatin digestion at 32°C with 5,000 units/ml of HaeIII for 20 minutes, while the other half remained on ice. For subsequent electroelution, beads were transferred into the wells of an 0.8% agarose gel and electrophoresed in ‘physiological’ buffer (containing 25 units/ml of RNase inhibitor) at 2 V/cm for 5 hours. Radioactivity was measured by precipitation of 250 μl aliquots on ice with trichloroacetic acid at a final concentration of 10%. All solutions used in labeling experiments were treated with diethylpyrocarbonate to eliminate RNases (Sambrook et al., 1989).
DNA purification and hybridization

To recover DNA from encapsulated nuclei, beads were digested overnight at 37°C with proteinase K (100 µg/ml) in the presence of 0.25% lithium dodecyl sulfate and subjected to electrophoresis in dialysis bags in 40 mM Tris, 5 mM Na-acetate, pH 8.0, 1 mM EDTA at 4°C for 3 hours. Yields were determined by ethidium bromide staining as 85-90%. Purification of DNA samples, Southern blotting and hybridization were performed by routine procedures (Sambrook et al., 1989). Briefly, 10 µg of purified DNA samples C, N, and Ns, samples E and M (total 10 µg), and samples Es and Ms (total 10 µg) were electrophoresed on 1% agarose gels in 36 mM Tris, 30 mM NaH2PO4, pH 8.0, 2 mM EDTA and stained with ethidium bromide. DNA fragments were vacuum-blotted onto BA 85 nitrocellulose filters (Schleicher and Schuell) in 20 DNA fragments were vacuum-blotted onto BA 85 nitrocellulose filters (Schleicher and Schuell) in 20× SSC or 1 M ammonium acetate, 0.02 M NaOH and hybridized overnight at 65°C in 4× SSC, 50 mM Na-phosphate, pH 6.5, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll. 100 µg/ml salmon sperm carrier DNA with radiolabeled probes. Probes were 32P-labeled by random hexanucleotide priming to a specific activity of ~1-3×108 cpm/µg DNA (Cerenkov counting) (random primed DNA labeling kit, Boehringer, Mannheim). The following probes were used: the 2,765 bp chicken lysozyme gene 5′ MAR fragment B-1-SacI (Phi-Van and Strätling, 1988), the 770 bp chicken lysozyme gene promoter fragment PstI containing the sequence between positions –489 and –281 (Dölle and Strätling, 1990), and the 657 bp Drosophila HinII-EcoRI fragment containing the Drosophila histone SAR (Mirkvitch et al., 1984). Filters were exposed to Kodak XAR-5 films at –80°C. DNA partitioning was quantitated by scanning of the films using a Bio-Rad model 620 video densitometer.

Electron microscopy

HD11 cells were encapsulated and processed to various points in the protocol as described. Beads were pelleted, fixed overnight at 4°C by resuspension in 3 volumes of physiological buffer containing 10% glutaraldehyde. Fixed beads were again pelleted and post-fixed with 1% osmium tetroxide in physiological buffer for 1 hour at 4°C. After initial dehydration in 35% ethanol, beads were mixed with 2% agarose (warmed to 60°C), briefly centrifuged, and the agarose blocks from the tips of the centrifuge tubes were continued to be dehydrated through a series of increasing ethanol concentrations that ended with two changes of 100% ethanol. Agarose blocks were then immersed in propylene oxide (two changes) and embedded in Epon. Thin sections (90 nm) were stained with 1% uranyl acetate for 20 minutes and with 1% lead citrate for 5 minutes and were then examined in a Philips EM 300 electron microscope at 60 kV.

RESULTS

Nuclear morphology of encapsulated HD11 cells during lysis, chromatin digestion and electroelution

The electron micrograph in Fig. 2A illustrates chicken HD11 myelomonocytes encapsulated in 0.5% agarose microbeads. Treatment with 0.5% Triton X-100 in physiological buffer (Jackson et al., 1988) followed by two additional washes in this buffer removed both cell and nuclear membranes and extracted most cytoplasm; the nucleus surrounded by collapsed cytoskeletal material remained (Fig. 2B). Cell lysis well preserved the structure of the nucleolus and its subcompartments, yet the chromatin has gained a coarser appearance and much heterochromatin has decondensed. (A case with less pronounced decondensation is the additionally electroeluted nucleus in Fig. 2C.) Both effects have been observed previously to occur after lysis with Triton X-100 (Jackson et al., 1988). The efficient treatment with Triton (three washes) probably solubilized essential nuclear components. As a further explanation, we note that the buffer used is certainly physiological in many important aspects but, nevertheless, lacks compounds that are normal nuclear constituents and may have functions in chromatin packaging but have been omitted due to potential deleterious effects (e.g. Ca2+) (Jackson et al., 1988). The rough nuclear circumference is most probably caused by the removal of the nuclear membranes. When lysed cells were electroeluted in physiological buffer without prior digestion, nuclei remained intact and changes in nuclear morphology relative to nuclei in lysed cells did not occur (Fig. 2C). In contrast, when chromatin was first digested with HaeIII (2,500 units per ml) for 45 minutes, nearly all of the chromatin was subsequently electroelutable (Fig. 2D). HaeIII was selected, since this enzyme as a 4-cutter cleaves frequently, and since we found that digestion of HaeIII in physiological buffer, in contrast to digestion with e.g. HinI, HindIII or BamHI, was as efficient as in the buffer recommended by the manufacturer (data not shown). Furthermore, we established in pioneering experiments, which quantitated the extent of digestion as a function of enzyme concentration and other parameters, that under the conditions used chromatin digestion with HaeIII was complete (data not shown; see next section). Three examples in Fig. 2D show that electroeluted nuclei were almost completely devoid of chromatin. Only three structures remained: the nuclear lamina, residual nucleoli and a loose nuclear network. This network may represent remaining chromatin fibers, a lattice of nucleoskeletal filaments or residual chromatin adhering on such filaments. A filamentous network with characteristics of intermediate filaments has been previously described in HeLa cell nuclei, which were either digested with HaeIII and electroeluted (Jackson and Cook, 1988) or were digested with DNase I and extracted with 0.25 M ammonium sulfate (He et al., 1990). Previous electron micrographs of encapsulated, partially digested and electroeluted HeLa cell nuclei showed clumps of chromatin attached to an intermediate filament-like nuclear skeleton (Jackson and Cook, 1988). As expected, our attempts to digest chromatin to completion and the almost complete removal of chromatin during electroelution made the emptied nuclei very fragile, despite them being protected by encapsulation. Thus digested, electroeluted nuclei routinely appeared deformed (Fig. 2D). Efficient digestion has been previously reported to result in nuclear collapse (Jackson and Cook, 1988). It can be prevented by incomplete digestion so that some chromatin remains after electroelution, or by prefixed prior to electroelution.

Chromatin DNA electroelutes from encapsulated and digested nuclei but nascent RNA does not

To quantitate the partitioning of bulk chromatin upon digestion and electroelution, DNA was purified from HaeIII-digested nuclei (N) of encapsulated HD11 myelomonocytes or DU249 hepatocytes, from the electroeluted fraction (E), and from the fraction (M) remaining in electroeluted nuclei. DNA fragments were displayed by agarose gel electrophoresis and stained with ethidium bromide (Fig. 3A and D). For both cell types, ~97% of the chromosomal DNA electroeluted from encapsulated and digested nuclei, confirming our electron microscope observations. In six independent experiments using HD11 cells, the...
HaeIII-digested DNA exhibited a mass average of 4.9 kb (variation 4.2-5.6 kb) and a number average of 2.9 kb. A mean of 3.3% (variation 2.4-4.5%) of nuclear DNA remained in nuclei after electroelution. This allows the calculation of an average loop size of 90 kb (variation 73-104 kb), which compares well with the average loop size in HeLa cells (86 kb) (Jackson et al., 1990).

Since it has been reported for human HeLa cells (Jackson and Cook, 1985) that nascent RNA in contrast to bulk chromatin resists electroelution, we attempted to reproduce this
Electroelutability of MAR/SARs

Observation for chicken HD11 cells. Following encapsulation, cellular DNA was uniformly labeled by overnight growth in medium containing [14C]thymidine, and cells were then incubated with [3H]uridine for 3 minutes to label nascent RNA. After lysis, encapsulated nuclei were digested briefly with HaeIII and electroeluted. Table 1 shows the results from three independent experiments. After limited digestion with HaeIII, 83-59% of chromatin DNA was electroeluted, while in undigested controls all DNA was retained. In contrast, only 0 to 28% of the pulse-labeled RNA was removed from digested nuclei by electroelution. Thus, under conditions that allow electroelution of the majority of the DNA, most of the nascent RNA was retained.

The variation among the experiments in the amount of nascent RNA retained in digested, electroeluted nuclei (72-100%) most likely resulted from residual RNase activities, though significant efforts were undertaken to inactivate endogenous and exogenous RNases (see Materials and Methods). In undigested nuclei all labeled RNA was refractory to electroelution, except for experiment 2 (only 50% retained). This exception is also most likely due to residual RNase activity, since in the digested portion of the sample 72% of the RNA was recovered after electroelution.

**The lysozyme gene 5′ MAR is electroelutable from encapsulated and digested nuclei**

We have previously shown that a 3 kb, AT-rich sequence located 8.5 kb upstream of the chicken lysozyme gene selectively binds to nuclear matrices prepared by DNase I digestion and extraction with high salt (Fig. 1) (Berezney and Coffey, 1974; Cockerill and Garrard, 1986; Phi-Van and Strätling, 1988). Consequently this sequence belongs to the diverse family of MAR sequences. In addition, the 5′ MAR sequence is selectively attached to the nuclear scaffold remaining after extraction with LIS and digestion with restriction enzymes (Mirkovitch et al., 1984; Phi-Van and Strätling, 1988). To
Table 1. Nascent RNA largely resists electroelution

<table>
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<th>Encapsulated cells</th>
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<th>Digested and electroeluted nuclei</th>
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<td></td>
<td>DNA</td>
<td>RNA</td>
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<td>RNA</td>
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<tr>
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<td>13,200</td>
<td>143,000</td>
<td>16,800</td>
<td>123,800</td>
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<tr>
<td>Exp. 3</td>
<td>2,450</td>
<td>2,620</td>
<td>1,440</td>
<td>1,490</td>
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DNA in encapsulated HD11 cells was uniformly labeled with [14C]thymidine, the RNA then pulse-labeled for 3 minutes with [3H]uridine. Following cell lysis, resulting encapsulated nuclei remained undigested or were digested with HaeIII. Either treatment was followed by electroelution. The numbers (mean of three measurements) give dpm per 250 μl of 50% bead suspension (1.0-1.5x10⁶ cells) for three representative experiments. Fractions given as percentage of the respective values in untreated nuclei are shown in brackets.

electroelute after HaeIII-digestion from nuclei of expressing as well as of non-expressing cells. It is important to note that electroelution was carried out at a chromatin digestion level, where the electroeluted chromatin encompassed 5’ MAR containing DNA fragments having sizes of 2.2-8.5 kb. Since the 5’ MAR itself is only 2.95 kb in length, most of these DNA fragments contained flanking non-MAR sequences. Therefore, the above conclusion can be extended by stating that the 5’ MAR chromatin as well as the immediately flanking chromatin are electroelutable from encapsulated and digested nuclei.

It is possible that, under conditions of complete digestion of nuclear chromatin with HaeIII, potential small differences in the electroelutability of bulk and 5’ MAR chromatin are not detectable. Complete digestion might also weaken a potential attachment of 5’ MAR chromatin to nuclear matrix. Therefore we chose milder conditions to digest HD11 nuclei with the aim that bulk chromatin would be only partially electroelutable. After digestion of encapsulated nuclei with 500 units and 1,000 units of HaeIII for 30 minutes, comparison of lanes N in Fig. 4A (500 units) and 4C (1,000 units) with lane N in Fig. 3A (2,500 units, 45 minutes) demonstrates that chromatin
Electroelutability of MAR/SARs

digestion under these conditions was incomplete. The milder conditions furthermore resulted in a decrease of the amount of DNA electroelutable: 71.9% of bulk DNA was electroeluted after digestion with 500 units, and 85.3% after digestion with 1,000 units.

Comparison of lanes N in Fig. 4B and D with lane N in Fig. 3B indicates that also the 5′ MAR chromatin was fragmented to a weaker extent under the milder conditions of HaeIII digestion. Scanning of lanes Es and Ms (Fig. 4B and D) in the original autoradiogram furthermore shows that after digestion with 500 units 30.2% of the 5′ MAR fragments remained in electroeluted nuclei, and that after digestion with 1,000 units 12.9% remained. Since these values are comparable to the remaining portions of bulk DNA, 5′ MAR fragments were not selectively retained in partially digested, electroeluted nuclei.

The lysozyme gene promoter and coding sequence are also electroelutable

In early studies on nuclear matrix composition, application of the method of DNase I digestion and high salt extraction gave rise to preparations that were enriched in transcribed sequences (e.g. see Hentzen et al., 1984; Jackson et al., 1981). We therefore extended our use of the agarose microbead technique on the lysozyme gene promoter and coding sequence. Encapsulated nuclei from expressing HD11 cells were digested with HaeIII, and purified secondarily digested DNA was Southern blotted and hybridized with a probe that detects a 1.0 kb fragment containing the promoter and the first 0.55 kb of the coding sequence (see map in Fig. 1). Consequently a single 1.0 kb band was visualized in lane Ns of the Southern blot in Fig. 3C.

After electroelution of encapsulated and digested nuclei from HD11 cells, the 1.0 kb promoter-coding sequence fragment was quantitatively recovered in the eluted fraction (compare lanes Es and Ms in Fig. 3C). In addition, by use of a cDNA probe we could show that also the remaining coding sequence of the gene completely partitioned into the eluted fraction (data not shown). We conclude that transcription of the lysozyme gene in HD11 cells does not impede electroelution of the chromatin encompassing the transcribed sequence and the promoter. Fig. 3F furthermore shows that also in non-expressing DU249 hepatocytes the 1.0 kb promoter-coding fragment quantitatively partitioned into the eluted fraction (lane Es). Finally, a comparison of lanes N in Fig. 3B and E with those in C and F indicate that the promoter region is more accessible to HaeIII digestion than the 5′ MAR encompassing chromatin, irrespective of whether the gene is transcribed or not. Possibly this reflects an elevated transcription-independent accessibility of specifically the HaeIII sites at the promoter rather than a general feature of the promoter chromatin.

Probing the Drosophila histone SAR by electroelution

SARs have been originally described as DNA elements that, after digestion of LIS-extracted nuclei, partition with a pelletable nuclear scaffold structure (Mirkovitch et al., 1984). The first described SAR is a 657 bp fragment contained in the AT-rich spacer that separates the histone H1 gene from the histone H3 gene in the tandemly repeated Drosophila histone gene cluster (see map in Fig. 5B) (Mirkovitch et al., 1984). Though SARs have been subsequently described for several other gene loci (Gasser and Laemmli, 1986), we selected the histone SAR to probe its electroelutability from encapsulated and digested nuclei, since it does not overlap with known regulatory sites or origins of DNA replication. Cells of the Drosophila embryonic Kc cell line were encapsulated, lysed, digested with HindIII, and electroeluted. The purified DNA was separated by gel elec-
trophoresis and stained with ethidium bromide (Fig. 5A, left panel). Lane N shows that cleavage of encapsulated Kc cell nuclei with Hinfl was much less efficient than that of chicken cell nuclei with HaeIII though both are frequent 4 bp cutters (see Fig. 3A and D, lanes N). Most probably, the severely depressed cleavage activity of Hinfl in ‘physiological’ buffer (see above) was responsible for the inefficiency of digestion. After electroelution of digested nuclei, 63% of nuclear DNA was recovered in the eluted fraction (Fig. 5A, left panel, lane E). From the fragment distribution in lane N, we estimate the molecular mass of the chromatin fragments resulting from Hinfl digestion as \( \sim 2 \times 10^7 \) to at least \( 5 \times 10^8 \). Likely, the partial electroelution of these fragments resulted from their enormous size.

Following Southern blotting, hybridization with a SAR probe visualized the SAR sequence in a heterogeneous collection of high molecular mass DNA fragments (lane N in the right panel of Fig. 5A). A calculation shows that these are contained in chromatin fragments of molecular masses between \(-1\) and \(10^7\). Thus the low level of digestion of the histone gene repeat cluster reflects the low digestion level of bulk DNA (see lane N in the left panel of Fig. 5A). The histone SAR is contained in a 692 bp Hinfl fragment (see map in Fig. 5B). This fragment was not visible in nuclear DNA that was not secondarily digested (Fig. 5A, right panel, lane N), but appeared as the sole fragment after secondary digestion (Fig. 5A, right panel, lane Ns). Upon electroelution of digested nuclei, 68% of the histone SAR containing chromatin partitioned into the eluted fraction (Es). In three independent experiments, 58-75% of the bulk DNA (mean 64.4%) and 63-76% of the SAR DNA (mean 69.0%) were electroeluted. Thus the degree of electroelutability of the histone SAR containing chromatin was comparable to that of bulk chromatin. Weak bands at 0.97 kb and 1.24 kb are visible in lane N (Fig. 5A, right panel). These most likely represent fusion fragments that additionally contain 274 bp from upstream or/and 277 bp from downstream (see map in Fig. 5B). We also noticed a greater than 20-fold variability in the signal intensities of the fusion fragments in the molecular mass range between 1.0 and 5 kb (lane N). Since the histone gene repeat is 5.0 kb in length, these fragments contain a single, complete copy of the SAR. Taking into account even the possibility that the strong bands represent doublets or triplets, we have to conclude that the noticed variability reflects great differences in the accessibility of the Hinfl sites in the histone repeat chromatin. The more accessible sites likely map to nucleosome linker regions, DNase I-hypersensitive sites and/or topoisomerase II-sensitive sites (Worcel et al., 1983; Käs and Laemmli, 1992).

**DISCUSSION**

**After removal of 97% of the chromatin the nuclear lamina, residual nucleoli and a nuclear network remain**

Chicken cells were encapsulated in agarose microbeads and lysed with Triton X-100 in a ‘physiological’ buffer. Resulting encapsulated nuclei were digested with a restriction enzyme and electroeluted using the same buffer. This technique, established by Cook et al. (Jackson et al., 1988), is considered to be a comparatively mild one, since encapsulation protects nuclei from gross physical mistreatment and greatly facilitates manipulations such as buffer changes, etc. Second, the use of a ‘physiological’ buffer preserves nuclear morphology and composition (for a more detailed discussion see Jackson et al., 1988). The changes we observed in lysed cells (a coarser appearance of chromatin and a partial decondensation of heterochromatin) most likely resulted from the use of Triton X-100. Very similar effects of Triton have been previously reported for encapsulated HeLa cells (Jackson et al.,...
RNA is associated with chromatin that is electroeluted last. This circumstance previously reported experiments that used encapsulated nuclei, in the experiments shown in Table 1 and also in most of the extracted nuclei (He et al., 1991). We emphasize, however, that a significant portion of the labeled RNA retained likely rep-resent nascent pre-rRNA, due to the high rate of transcription of incorporation were visualized by antibodies directed against HeLa cell nuclei (Jackson and Cook, 1985). In a more recent study, nascent RNA has also been reported for encapsulated, EcoRI-digested HeLa cell nuclei (Jackson and Cook, 1985). In a more recent study, nascent RNA was pulse-labeled with Br-UTP and sites of incorporation were visualized by antibodies directed against Br-RNA and a second fluorescent antibody (Jackson et al., 1993). Sites of transcription localized to a network of foci spreading throughout the nucleoplasm. Significantly, these focal sites were found to be refractory to DNA digestion and subsequent electroelution. These results argue that nascent RNA is associated directly or indirectly with an underlying nucleoskeletal structure. Using immunoelectron microscopy, also heterogeneous nuclear ribonucleoprotein particles have been previously shown to be tightly associated with the filament network in DNase I-digested and 0.25 M ammonium sulfate-extracted nuclei (He et al., 1991). We emphasize, however, that in the experiments shown in Table 1 and also in most of the previously reported experiments that used encapsulated nuclei, chromatin was only partially electroeluted. This circumstance allows us to consider the alternative possibility that nascent RNA is associated with chromatin that is electroeluted last.

Digestion of encapsulated chicken nuclei followed by electroelution removed 97% of the chromatin. Solely, the nuclear lamina, residual nucleoli and a loose nuclear network remain. In previous studies this network, though yielded from less efficiently digested nuclei, has been extensively investigated. It represented clumps of chromatin attached to a network of intermediate filament-like fibers (Jackson and Cook, 1988). A three-dimensional anastomosing nuclear network also became visible by whole mount microscopy in unextracted cells and in cells extracted with 0.25 M ammonium sulfate (Capco et al., 1982). A core network of branched filaments with diameters of 9 and 13 nm was best visible after a final extraction with 2 M NaCl (He et al., 1990). A very recent immunocytochemical study showed evidence that lamin-like proteins are constituents of the nuclear network (Hozák et al., 1995). The electroeluted nuclei in Fig. 2 appear to be relatively empty, since the remaining network stained only weakly. It has been previously noted that conventional thin section electron microscopy yields only a poor image of the nuclear matrix network (Capco et al., 1982). In the residual nucleoli the gross nucleolar morphology was very often preserved, and fibrillar centers, dense fibrillar components and a granular component are discernible.

Under conditions where most chromatin was electroelutable from digested chicken cell nuclei, nascent RNA was largely resistant to electroelution. Since GC-rich (70%) pre-rRNA labels poorly with [3H]uridine and since the [3H]uridine pulse was short (3 minutes), a major portion of the labeled RNA retained represents pre-mRNA. Pioneering experiments in the 1960s already had established that, when animal cells are allowed to incorporate [3H]uridine for 5 minutes or less, most of the labeled RNA is heterogeneous in size and lies outside the nucleolus (Darnell, 1968). Furthermore, the average nucleotide composition of the labeled RNA is similar to that of DNA and very different from that of pre-rRNA. We emphasize, however, that a significant portion of the labeled RNA retained likely represents nascent pre-rRNA, due to the high rate of transcription in nucleoli (Wachtler et al., 1990). This would be consistent with the excellent preservation of nucleolar morphology after digestion and electroelution. A selective retention of nascent RNA has also been reported for encapsulated, EcoRI-digested HeLa cell nuclei (Jackson and Cook, 1985). In a more recent study, nascent RNA was pulse-labeled with Br-UTP and sites of incorporation were visualized by antibodies directed against Br-RNA and a second fluorescent antibody (Jackson et al., 1993). Sites of transcription localized to a network of foci spreading throughout the nucleoplasm. Significantly, these focal sites were found to be refractory to DNA digestion and subsequent electroelution. These results argue that nascent RNA is associated directly or indirectly with an underlying nucleoskeletal structure. Using immunoelectron microscopy, also heterogeneous nuclear ribonucleoprotein particles have been previously shown to be tightly associated with the filament network in DNase I-digested and 0.25 M ammonium sulfate-extracted nuclei (He et al., 1991). We emphasize, however, that in the experiments shown in Table 1 and also in most of the previously reported experiments that used encapsulated nuclei, chromatin was only partially electroeluted. This circumstance allows us to consider the alternative possibility that nascent RNA is associated with chromatin that is electroeluted last.

The procedures used here, in particular the use of encapsulated cells and of near-physiological buffer conditions, are thought to be comparatively mild ones. We also note that the electroelution conditions (2 V/cm) were milder than the electrophoretic conditions (~12 V/cm) used in conventional band shift experiments (Varshavsky, 1987). Band shift experiments are frequently employed to detect specific protein-DNA interactions with high sensitivity. Therefore, it is unlikely that potential MAR-nuclear matrix interactions are disrupted by the electroelution conditions. Nevertheless, we cannot exclude the possibility that the cell lysis, the chromatin digestion step, or other aspects of our procedures lead to alterations in chromatin structure that finally result in changes of a hypothetical asso-ciation of the 5′ MAR to a nuclear matrix. In this context, the coarser appearance of chromatin in lysed cells relative to intact cells may be interpreted as an induced change in chromatin structure.

To approach physiological buffer conditions, we restricted our attempts to the use of the near-physiological buffer recommended by Cook et al. (Jackson et al., 1988). Specific attempts to stabilize nuclear matrix structure, e.g. by heat treatment, incubation with sodium tetrathionate or with Cu2+, were not undertaken, since it is not clear whether these treatments may produce perturbations of chromatin organization. Previous work presented evidence that either incubation of isolated nuclei at 37°C or exposure to Cu2+ (at 4°C) was necessary to obtain so-called matrix I structures that contained an internal nuclear network (Mirkovitch et al., 1984). It was thought that metalloproteins are critical constituents of the nuclear matrix (scaffold). Furthermore, early studies showed that sulfhydryl group oxidizing agents, such as sodium tetrathionate promote the generation of an extensive salt- and DNase I-insoluble intranuclear structure (Kaufmann et al., 1981). Though we haven’t used any of these reagents and despite the fact that the ‘physiological’ buffer contained 1 mM...
DTT, we obtained a loose internal nuclear network. In previous reports thiol reagents and metal chelators have been shown to lead to a removal of the nuclear matrix (scaffold) (Kaufmann et al., 1981; Lewis and Laemmli, 1982). Possibly this effect requires that chromatin is disaggregated by high salt or LIS and does not occur, when chromatin is more carefully treated by restriction enzyme digestion.

**Drosophila histone SAR chromatin electroelutes to same extent as bulk chromatin**

Scaffold attached regions (SARs) have been biochemically defined as sequences that remain bound or bind to nuclei, which have been extracted with the chaotrope LIS (Mirkovitch et al., 1984). LIS extracts the majority of the histones as well as non-histone proteins. Here we have shown that *Drosophila* histone SAR chromatin electroelutes from encapsulated and *Hinfl*-digested nuclei to the same extent as bulk chromatin. We conclude that the histone SAR is not permanently attached to a nuclear scaffold. However, if a high on-off rate of attachment is assumed, the elutability could also be explained by the long time period (5 hours) required for electroelution. In previous studies, an ectopic copy of the *Drosophila fushi tarazu* SAR was bound to LIS-extracted nuclei, e.g. conform to the definition of a SAR (Eggert and Jack, 1991). On the other hand, the ectopic SAR was electroeluted from encapsulated nuclei as readily as a non-SAR fragment. It was concluded that this SAR is not attached to a nuclear scaffold in vivo.

**Domain organization and nuclear matrix**

The chicken lysozyme gene domain, illustrated in Fig. 1, is an impressive example of a functional genetic unit. It includes all known regulatory elements required in *cis* (Bonifer et al., 1991). The domain is defined by an elevated DNase I sensitivity and is limited by sequences that fulfills the definition of MARs as well as that of SARs (Jantzen et al., 1986; Phi-Van and Strätling, 1988). These domain boundaries (=MARs =SARs) have been further shown to have important biological effects. On the flanks of stably integrated transgenes the 5' MAR elevates gene expression and dampens position effects. On the other hand, the ectopic SAR was electroeluted from encapsulated nuclei as readily as a non-SAR fragment. It was concluded that this SAR is not attached to a nuclear scaffold in vivo.

**REFERENCES**


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