The Scaffold/Matrix Attachment Region Binding Protein hnRNP-U (SAF-A) Is Directly Bound to Chromosomal DNA *in Vivo*: A Chemical Cross-Linking Study†

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ABSTRACT: The protein heterogeneous nuclear ribonucleoprotein U (hnRNP-U, also known as scaffold attachment factor A, SAF-A) is an abundant component of hnRNP particles and of the nuclear matrix. Previous experiments have demonstrated that, in vitro, hnRNP-U specifically binds to scaffold/matrix attachment (S/MAR) region DNA elements and could thus be involved in higher order chromatin structure. In this paper we report on the use of chemical cross-linking to investigate whether the protein is also bound to DNA in vivo, which is a prerequisite for its presumed function in chromatin loop formation. We have improved published methods for cross-linking proteins to DNA with the aim to minimize unspecific fixation and possible contamination with RNA binding proteins. Our protocol is based on a limited cross-linking of living human cells with formaldehyde, followed by the purification of DNA/protein complexes by two consecutive cesium chloride density gradient centrifugations. Analysis of the protein constituents of these complexes shows a specific subset of cross-linked proteins with the histones as major components. By western blotting, we demonstrate that hnRNP-U is efficiently cross-linked to DNA under experimental conditions that yield DNA/protein complexes with a buoyant density equivalent to that of native chromatin. Dimethylsulfate cross-linking and limited protease digestion of the complexes was used to establish that hnRNP-U is bound directly to DNA and not via cross-linking to other proteins. This is the first direct demonstration of the in vivo DNA binding of a S/MAR specific protein and suggests a structural role of hnRNP-U in chromatin organization.

The eukaryotic nucleus is a highly ordered organelle specialized in nucleic acid metabolism. Much of the organization of the nucleus is presently attributed to a proteinaceous framework, termed nuclear scaffold or matrix, that fills the nuclear interior and specifically interacts with certain A+T-rich DNA fragments, the scaffold/matrix attachment regions (S/MARs). Current models interpret S/MARs as the bases of chromatin loops that form to partition the genome into structural or functional subcompartments (Garrard, 1990; Gasser & Laemmli, 1987). Apart from this structural role, S/MARs might also have regulatory functions, as they are frequently found in the vicinity of enhancer elements (Cockerill & Garrard, 1986; Cockerill et al., 1987; Gasser & Laemmli, 1986) and can stimulate the expression of heterologous reporter genes when integrated into the genome (Phi-Van & Strätling, 1996; Phi-Van et al., 1990; Poljak et al., 1994; Stief et al., 1989; Xu et al., 1989). At present, however, the exact function of S/MARs in vivo is not well understood. Additionally, the mechanism by which the nuclear scaffold recognizes the S/MAR DNA elements has not been clarified convincingly. It has been proposed that the nuclear scaffold contains proteins that specifically recognize unusual DNA structures often found in S/MARs, such as DNA bends (Homberger, 1989; von Kries et al., 1990), narrow minor grooves (Käs et al., 1989), or the single-stranded status of "unwinding elements" (Bode et al., 1992; Kay & Bode, 1994; Kohwi-Shigematsu & Kohwi, 1990). Further insight into the underlying mechanisms, however, is only possible by identifying and characterizing these proteins in molecular detail. Several recent studies have reported on proteins that specifically, although in most cases not exclusively, bind to S/MAR DNA elements in vitro and could thus be involved in chromatin higher order structure. These specific S/MAR binding proteins include abundant ubiquitous proteins like histone H1 (Izaurralde et al., 1989), topoisomerase II (Adachi et al., 1989; Käs & Laemmli, 1992), hnRNP-U (also known as scaffold attachment factor A, SAF-A; Fackelmayer et al., 1994; Kiledjian & Dreyfuss, 1992; Romig et al., 1992), SAF-B (Renz & Fackelmayer, 1996), lamin B1 (Luderus et al., 1992; Luderus et al., 1994), p53 (Deppert, 1996), HMG I/Y (Zhao et al., 1993), ARBP (von Kries et al., 1991), and nucleolin (Dickinson & Kohwi-Shigematsu, 1995) but also proteins that are expressed primarily in certain cell types, like SATB1 (Dickinson et al., 1992; Nakagomi et al., 1994) or p114 (Yanagisawa et al., 1996). It is not yet clear which of these proteins are bound to S/MAR DNA in vivo, but it appears that S/MAR−protein interactions are at least partially dependent on cell type and transcriptional activity.

In our efforts to examine the interaction between genomic DNA and the nuclear scaffold, we have previously identified four specific S/MAR-DNA binding proteins and have named them scaffold attachment factors A through D (SAF-A to SAF-D; Romig et al., 1992). More recently, we have characterized the two most abundant proteins, SAF-A and SAF-B, in molecular detail (Fackelmayer et al., 1994; Fackelmayer & Richter, 1994a,b; Renz & Fackelmayer, 1996). While SAF-B is a novel protein with no significant homology to any known protein, SAF-A was demonstrated to be identical with hnRNP-U, a protein thought to be involved in RNA metabolism at the stage of pre-mRNA

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packaging (Kiledjian & Dreyfuss, 1992). Indeed, hnRNP-U also binds to RNA in vitro (Fackelmayer & Richter, 1994b) but prefers DNA over RNA of the same sequence (Fackelmayer & Richter, 1994a). Although hnRNP-U is a general nucleic acid binding protein, its strong affinity toward S/MAR DNA is demonstrated in competition experiments (Romig et al., 1992). This high binding specificity has been independently confirmed by others (Tsutsui et al., 1993; von Kries et al., 1994).

The results of previous studies indicate that hnRNP-U/SAF-A is a bifunctional protein, with one role in pre-mRNA packaging and the other in the higher order organization of chromatin. Consistent with this second function, approximately 50% of the total hnRNP-U is structurally fixed in the nucleus by a tight, salt-stable binding to the nuclear scaffold (Fackelmayer et al., 1994; Mattern et al., 1996). The remaining 50% of the protein distributes equally between a soluble population and a DNase I extractable population (Fackelmayer et al., 1994; unpublished observations).

Many details on the binding of hnRNP-U to nucleic acids have been investigated using the purified protein from human cells. Although these studies gave conclusive information on nucleic acid preference and specificity, the question on the biological significance of these findings could not be answered. In particular, it appeared possible that hnRNP-U is bound to RNA in vivo whereas DNA binding is a property that can only be observable in vitro. To resolve this issue, we have performed experiments to study the binding of hnRNP-U to DNA in vivo. In a previous publication we have shown that hnRNP-U can be cross-linked to DNA by irradiation of cells with UV light (Fackelmayer et al., 1994), compatible with the finding that a certain subpopulation of hnRNP-U can be extracted from nuclei by nuclease digestion. The amount of binding observed with UV cross-linking, however, was very low, as has been noted before by others for other proteins (Gilmour & Lis, 1984, 1987). As DNA binding is a prerequisite for the protein's presumed function in attaching chromatin to the nuclear scaffold, it appeared necessary to independently confirm the DNA binding of hnRNP-U in living cells by more efficient cross-linking techniques. To this end, we used chemical cross-linking with formaldehyde (methanal). Starting from published cross-linking procedures, we have devised a modified method that minimizes the formation of unspecific cross-links and largely excludes contamination with non-cross-linked material. After a limited treatment of living cells with formaldehyde, resulting DNA/protein complexes were purified over two cesium chloride gradients and analyzed for their protein components after cross-link reversal. We present evidence that, by use of this method, hnRNP-U can be cross-linked to DNA in an amount of approximately 20% of the total cellular hnRNP-U. Thus, we can safely conclude that a significant amount of hnRNP-U is bound to DNA in vivo. Additionally, our protocol should be useful for the study of other DNA binding proteins.

MATERIALS AND METHODS

Cell Culture and DNA/RNA Labeling in Vivo. HeLa S3 cells were cultivated on 145 mm plastic dishes in Dulbecco's modified Eagle's (DME) medium with 5% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. For DNA or RNA labeling, the medium of semiconfluent plates was replaced by 20 mL of medium with 15 μCi of [*methyl*-^{3}H]thymidine or 15 μCi of [*methyl*-^{3}H]uridine (Amersham), respectively, and the cells were incubated for 24 h under normal culture conditions.

Formaldehyde Cross-Linking in Vivo. Five dishes with HeLa cells, one of which contained labeled cells, were washed once with 37 °C warm phosphate-buffered saline (PBS), aspirated to near dryness, and incubated for 4 min at 37 °C in 20 mL of DME medium (without serum) containing 1% formaldehyde (freshly added from a 37% stock solution, Merck). No quenching of the cross-linking reaction, e.g., with glycine buffers, was performed, as initial experiments had shown this step to be unnecessary.

Preparation of DNA/Protein Complexes. Cross-linked cells were rinsed thoroughly with cold PBS twice, aspirated, scraped off the petri dishes in 10 mL of PBS with a rubber policeman, and centrifuged for 5 min at 750*g* in a Sorvall SS34 rotor. After resuspension in 10 mL of RSB (10 mM Tris-HCl, 10 mM NaCl, and 3 mM $MgCl_2$, pH 8.0), cells were homogenized by 15 strokes in a chilled Dounce homogenizer and nuclei were collected by centrifugation (8 min, 750*g*). After nuclei were washed twice with RSB, many unbound proteins were extracted in buffer E (10 mM Tris-HCl, 10 mM $Na_2S_2O_5$, 1 M NaCl, 0.1% NP-40, 1 mM EDTA-KOH, and 0.5 mM PMSF, pH 8.0). This extraction step is tolerated by cross-linked nuclei (in contrast to non-cross-linked control nuclei that are lysed and form a highly viscous solution) and greatly improves the efficiency of DNA/protein complex purification. After extraction, nuclei were pelleted as above, resuspended in 2.7 mL of buffer E with only 0.1 M NaCl, and lysed by the addition of 0.3 mL of 20% sodium sarkosyl solution. The sample was then carefully layered over a preformed CsCl step gradient in a SW40 polyallomer ultracentifugation tube. The gradient consisted of 3 mL of 1.75 g/mL CsCl solution, 3.5 mL of 1.5 g/mL CsCl solution, and 3 mL of 1.3 g/mL CsCl solution; all CsCl solutions were prepared in 20 mM Tris-HCl, 1 mM EDTA-KOH, and 0.5% sodium sarkosyl. The gradient was centrifuged for 24 h at 37 000 rpm in an SW40 rotor at 20 °C and then fractionated from the top in 18 aliquots of 700 μL each. DNA/protein complexes sediment at a density of approximately 1.4 g/mL and are usually seen as a slightly turbid band by visual inspection of the gradient. Aliquots of the fractions were sheared by brief sonication, and the density of individual fractions was determined by refractometry. Relative DNA concentrations were measured by liquid scintillation counting, and DNA-containing fractions were pooled. For RNase digestion, pooled fractions were dialyzed against 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM EDTA before 5 μg of DNase-free RNase (Boehringer Mannheim) was added. After 30 min at room temperature, solid CsCl was added to a density of 1.5 g/mL in a final volume of 5 mL, and the sample was recentrifuged for 72 h at 40 000 rpm in an SW55 rotor at 20 °C. The gradient was fractionated into 18 aliquots of 280 μL each and analyzed as above. DNA/protein complexes sediment as a highly viscous aggregate that can be taken out by aspiration with a wide-bore pipette tip after removal of the gradient fractions above the complex band. The DNA-containing fraction was briefly sonified, desalted by gel fitration on Sephadex G50 columns (1-mL volume), concentrated by precipitation with TCA (25% final concentration), and dissolved in 500 μL of SDS−PAGE sample buffer.

Cross-links were cleaved by incubation in a boiling water bath for 10 min, and samples were analyzed on SDS−polyacrylamide gels.

DMS Treatment of DNA/Protein Complexes. Additional cross-linking with dimethylsulfate (DMS) was done exactly as described by Bavykin et al. (1993), using sonified, desalted DNA/protein complexes from a second gradient as starting material.

Limited Proteolysis. The highly viscous, purified DNA/protein complexes were removed from a second gradient, washed three times for 5 min each in TE buffer, and sonified. The protein concentration of the sample was determined, and proteolysis was performed in the presence of 0.5% SDS and low amounts of proteinase K (Boehringer Mannheim). Titration experiments were performed with protease:protein ratios of 1:100 to 1:30 000 to find optimal limited degradations. After 30 min at room temperature, protease digestion was stopped by the addition of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 1 mM, samples were centrifuged on an isopycnic CsCl gradient as described above and analyzed by SDS−PAGE and western blotting.

Other Methods. SDS−PAGE of proteins was performed according to Laemmli (1970); the gels were stained with Coomassie brilliant blue as described by Sambrook et al. (1989). Western transfer was performed according to Towbin et al. (1979) with affinity-purified polyclonal serum (Harlow & Lane, 1988), peroxidase-coupled secondary antibodies (Sigma), and enhanced chemiluminiscent detection (ECL, Amersham). Protein concentrations were determined using the Bio-Rad protein assay reagent with bovine serum albumin as standard.

RESULTS

Formaldehyde (methanal, HCHO), has repeatedly been used in vivo and in vitro to introduce covalent bonds between interacting proteins or between proteins and nucleic acids. However, published procedures for cross-linking with formaldehyde differ widely, ranging from 8 min at 37 °C with 1% formaldehyde (Solomon et al., 1988) to 4 days at 4 °C with 1% formaldehyde (Orlando & Paro, 1993). Using the conditions described by Solomon et al. (1988) as a starting point, we first established conditions that yield DNA/protein complexes with a density equivalent to that of native chromatin [approximately 1.4 g/mL (Hancock, 1974)]. These complexes can be considered to represent the physiological situation in the best possible way and should be suitable for examining the binding of hnRNP-U to DNA in vivo. To estimate the degree of cross-linking under different conditions, DNA/protein complexes were extracted from formaldehyde-treated cells by lysis with detergent and centrifuged on preformed cesium chloride gradients to determine their buoyant density (Figure 1). For analytical purposes, DNA had been uniformly labeled in vivo with low amounts of [^{3}H]thymidine prior to the addition of formaldehyde. The DNA concentration of indivual fractions of the gradient, and hence the position of DNA/protein complexes, was determined by liquid scintillation counting. A systematic variation of incubation time and formaldehyde concentration shows that increasing either one of these parameters results in a progressive shift of the density of DNA/protein complexes from that of free DNA (1.66 g/mL) to that of free protein (1.3 g/mL). Interestingly, we have never

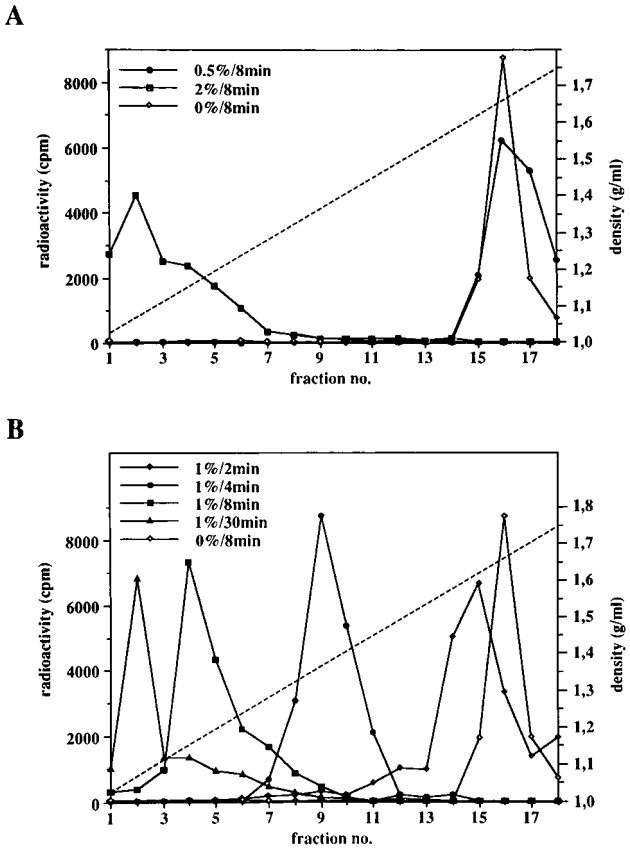

FIGURE 1: Optimization of in vivo cross-linking with formaldehyde. Cells containing ^{3}H-labeled DNA were treated with different concentrations of formaldehyde for a fixed time (A) or with a fixed concentration of formaldehyde for different times (B). After cross-linking, nuclei were prepared, lysed, and centrifuged on preformed cesium chloride gradients. DNA content and density of all fractions were assessed by liquid scintillation counting and refractometry, respectively. The dashed line represents an average density gradient.

observed free DNA under conditions that produce cross-links. This result indicates that, if cross-linking occurred, it involved the entire genome. Complexes with a density of 1.42 g/mL, corresponding to a mass ratio of 1:1 (protein:DNA) characteristic for native chromatin, were achieved by treatment of cells with 1% formaldehyde for 4 min at 37 °C. Consequently, we have used this treatment for all experiments reported below (except for Figure 4, see below).

Centrifugation over CsCl gradients is also a useful method for the purification of DNA/protein complexes. Purified complexes can then be de-cross-linked by boiling in SDS−PAGE sample buffer—a treatment that cleaves the methylene bonds introduced by formaldehyde—and analyzed for their protein components by gel electrophoresis [e.g., Jackson (1978) and Solomon et al. (1988)]. Thus, the presence of a given protein in DNA/protein complexes is a strong indication of whether it had been bound to DNA in vivo. As exemplified for a sample that had been cross-linked for 4 min, however, a single centrifugation is insufficient to remove free, non-cross-linked proteins that contaminate the DNA/protein complexes and impair gel electrophoretic analysis (Figure 2A,B). These contaminating proteins are efficiently eliminated by a second, isopycnic centrifugation over CsCl, yielding purified DNA/protein complexes well separated from free proteins (Figure 2C,D). Gel electrophoresis after cross-link reversal in sample buffer shows that only a subset of proteins is present in these complexes, in

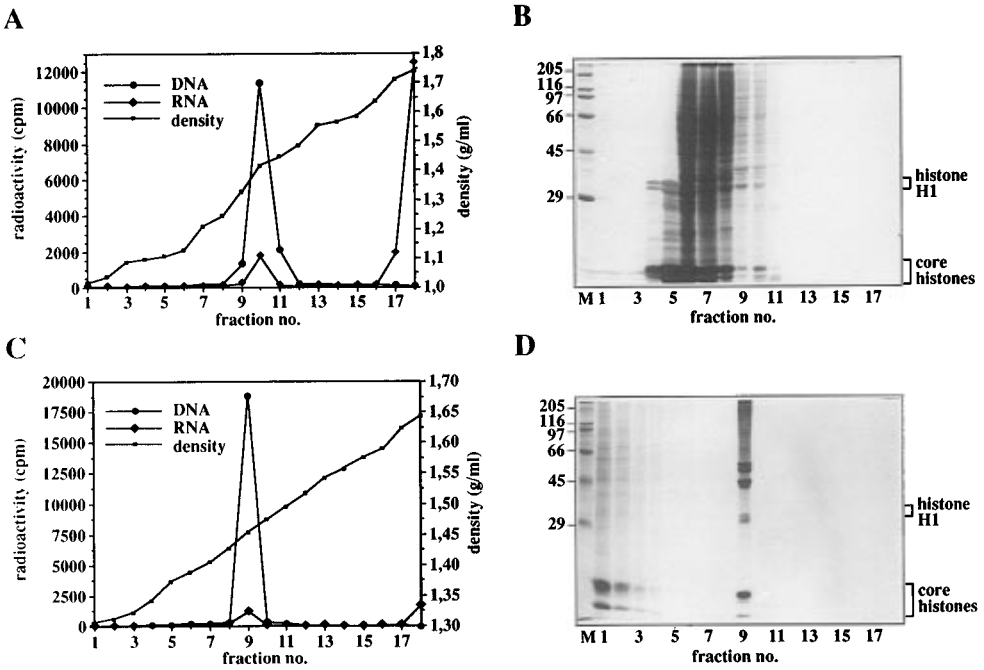

FIGURE 2: Purification of DNA/protein complexes by equilibrium centrifugation. Equilibrium centrifugation of DNA, RNA, and proteins was performed in two consecutive cesium chloride gradients. To determine the distribution of both nucleic acids, cells were labeled for 24 h with either [^{3}H]thymidine or [^{3}H]uridine before cross-linking. Lysed nuclei were centrifuged on preformed gradients and individual fractions were tested for DNA or RNA by liquid scintillation counting (A) and for proteins by SDS−PAGE and staining with Coomassie blue (B). For further purification, fractions 9−11 were pooled, recentrifuged on an isopycnic cesium chloride gradient, fractionated, and analyzed as above (C, D). Note that bulk RNA appears at the bottom of the first gradient without cross-linked proteins (panels A and B, fractions 17 and 18). The density gradients shown in panels A and C are from an experiment with labeled DNA. The density distribution for the experiment with labeled RNA was very similar, so that both experiments were combined in this figure.

contrast to the large number of proteins present in the unbound fractions. Core histones and histone H1 represent the majority of proteins in these complexes, whereas other proteins, mostly with molecular masses greater than 50 kDa, are present in lower amounts. This result is expected for chromatin and indicates that cross-linking under our experimental conditions preserves well the chromatin composition of the living cell, without artifactual fixation of other proteins (for a further proof of cross-linking specificity, see Figure 4). Interestingly, three to four strong protein bands other than that of the histones are seen in a Coomassie-stained gel with apparent molecular masses of 27 [seen as a faint band in Figure 2D but better visible in other experiments; see also Jackson and Chalkley (1981)], 43, 50, and 55 kDa (Figure 2D). The identity of these proteins is not yet known.

In the experiments reported in Figure 2, we have also addressed the question whether RNA is a component of the purified complexes. This control experiment was necessary to rule out that proteins seen in the complexes are interacting with RNA rather than with DNA, which could be the case for hnRNP-U that binds to both nucleic acids. For the experiment, RNA was labeled in vivo by [^{3}H]uridine prior to cross-linking, instead of labeling DNA with [^{3}H]thymidine as in our other experiments. Samples were then prepared and analyzed as described above. We find that the major fraction of RNA pellets in the first gradient and is apparently not complexed with proteins (Figure 2B, fractions 17 and 18). A small fraction of the RNA, however, cosediments with DNA/protein complexes in the first and in the second gradient. We interpret this cosedimenting RNA as primary transcripts that had been cross-linked to transcriptionally active chromatin. Treatment with RNase A prior to the second centifugation removed this RNA fraction with no detectable effect on the buoyant density of the complexes (data not shown). Thus, all experiments reported below were carried out on RNase-treated samples to rule out contaminations with proteins fixed in the complexes due to their interaction with RNA.

To find out whether hnRNP-U is a component of purified DNA/protein complexes, we performed western blotting experiments with the fractions of a second gradient (Figure 3). We expect that, if hnRNP-U is bound to DNA in vivo, a protein peak should be evident in the fractions with DNA/protein complexes. Indeed, this is observed (Figure 3, fraction 8 and 9). Additionally, unbound hnRNP-U is detectable in the top fractions of the gradient, where a control protein, rabbit IgG, sediments as a marker for non-cross-linked proteins. In a time course experiment equivalent to the one in Figure 1B we find that significant amounts of hnRNP-U become detectable in DNA/protein complexes after only 2 min of cross-linking (Figure 4, upper panel), and increased thereafter with the same rate at which histones are linked to DNA (Figure 4, lower panel). This suggests that hnRNP-U may be an intimate component of cross-linked chromatin. As the set of cross-linked proteins is limited to histones and few other proteins (compare with Figure 2), cross-linking appears to be highly specific in the first minutes of incubation. Longer incubation times lead to a further increase of hnRNP-U or histones by a factor of approximately 4, mainly because the density of DNA/protein complexes approaches the density of free proteins so that purification becomes insufficient (compare Figures 1 and 2) and the complexes are increasingly contaminated with non-cross-linked proteins.

To determine the fraction of the total cellular hnRNP-U cross-linked to DNA/protein complexes after limited cross-

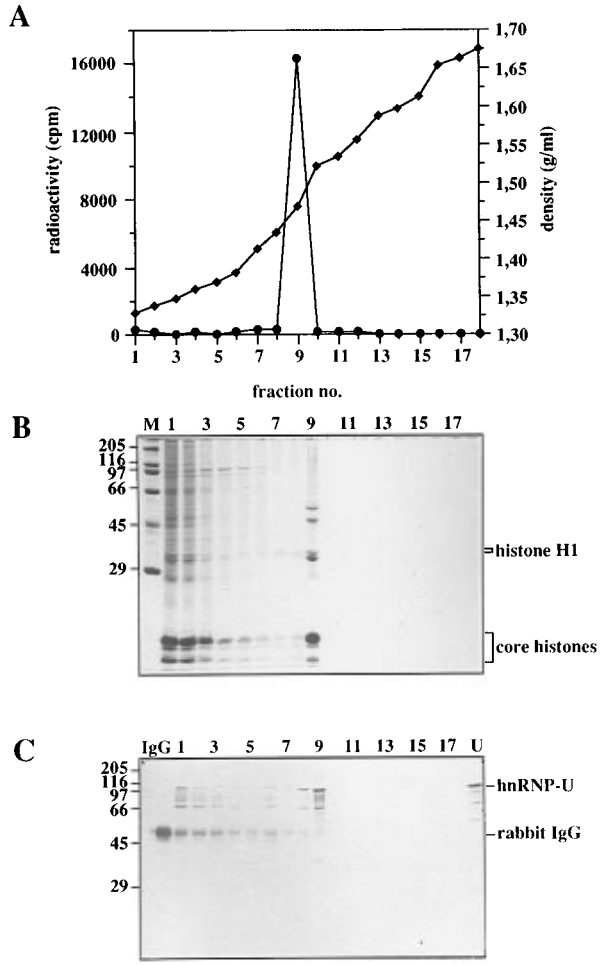

FIGURE 3: hnRNP-U is a component of DNA/protein complexes. DNA/protein complexes were purified from formaldehyde-treated treated cells by centrifugation over two consecutive CsCl gradients. Aliquots of all fractions of the second gradient (A) were analyzed for their protein content by SDS−PAGE and staining with Coomassie blue (B) or by western blotting of an identical gel, for the localization of hnRNP-U in the gradient (C). Note that hnRNP-U cosediments with bulk DNA and the histones.

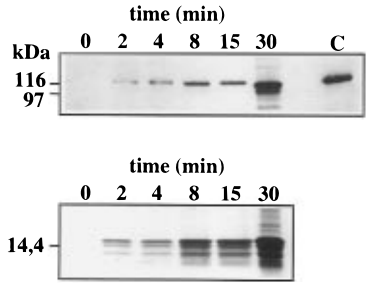

FIGURE 4: Time course experiment on the cross-linking of hnRNP-U to DNA. DNA/protein complexes were purified from cells treated with formaldehyde for increasing times. Equivalent aliquots of the fractions containing the DNA peak were analyzed for their content of hnRNP-U by western blotting (upper panel, 8% polyacrylamide gel; lane C shows an aliquot of purified protein as a positive control) or for their content of histones by Coomassie staining of SDS gels (lower panel, 15% polyacrylamide gel).

linking (Figure 5), we purified complexes from 5×10^6 cells (exposed to 1% formaldehyde for 4 min) and processed them for SDS−PAGE. Cross-linked hnRNP-U was quantified on the same gel with known aliquots of total cell extracts from the same number of untreated cells and a known amount of purified protein. Visual inspection and densitometric evaluation of the gel indicates that the cross-linked fraction

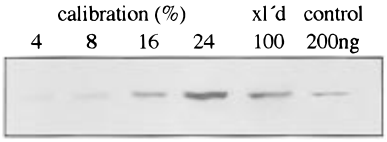

FIGURE 5: Quantification of cross-linked hnRNP-U. The amount of hnRNP-U cross-linked to DNA in vivo was quantified by western blotting of DNA/protein complexes from 10^5 treated cells (xl'd, 100%), along with increasing amounts of total cell extract from the same number of untreated cells (calibration) or a known amount of purified protein (control, 200 ng).

corresponds to approximately 20% of the total cellular hnRNP-U or 4×10^5 hnRNP-U molecules/nucleus [given a total of 2 million hnRNP-U molecules/nucleus (Fackelmayer & Richter, 1994b)]. The same result is obtained by comparison with a known amount of purified protein (Figure 5, control lane).

The stable association of hnRNP-U with DNA/protein complexes clearly demonstrates that the protein can be chemically cross-linked to chromatin and must be in close contact with DNA in vivo. However, formaldehyde is known to cross-link proteins to DNA both directly and indirectly, i.e., through the interaction with other proteins (Solomon et al., 1988). It was, therefore, of interest to determine whether hnRNP-U is directly bound to DNA. We have used two independent approaches to address this question. First, we have applied a published method for converting methylene bonds to more stable covalent bonds by treatment with dimethylsulfate (DMS; Bavykin et al., 1993). These bonds occur through formation of a Schiff base between ϵ-amino groups of lysine residues and a reactive aldehyde group that is formed by opening of the deoxyribose ring after methylation at purine bases and depurination of DNA. After reduction with sodium borohydride, covalent DNA−protein bonds resist boiling in SDS-containing buffers. Thus, a treatment of DNA/protein complexes with DMS allows to discriminate between a direct and an indirect binding of hnRNP-U to DNA, by determining whether the protein can be detached from DNA by standard de-cross-linking in SDS−PAGE sample buffer. We find that, in contrast to control samples treated in the same way without DMS, a certain amount of hnRNP-U shifts to a high molecular weight form that does not enter the SDS gel (Figure 6A). As DMS cross-linking is specific for DNA/protein bonds due to its unique reaction pathway (Bavykin et al., 1993), this gel shift indicates that hnRNP-U is directly bound to DNA. As a negative control, we determined under identical conditions the fate of hnRNP-A1 (Figure 6B), a protein not expected to be bound to DNA in vivo. We found indeed that hnRNP-A1 could not be cross-linked to high molecular weight material by treatment with DMS. We want to point out, though, that experiments of the type shown in Figure 6A must be performed under carefully controlled conditions because the excessive handling of the samples prior to their analysis is a potential source of error.

We have therefore used a second approach to independently demonstrate a direct binding of hnRNP-U to DNA. This approach is based on the idea that limited proteolysis of DNA/protein complexes should remove all proteins from the complexes that are indirectly linked to DNA, while the fragments of directly bound proteins should remain on the DNA. By titration of the amount of proteinase K added to the complexes, we found conditions under which statistically

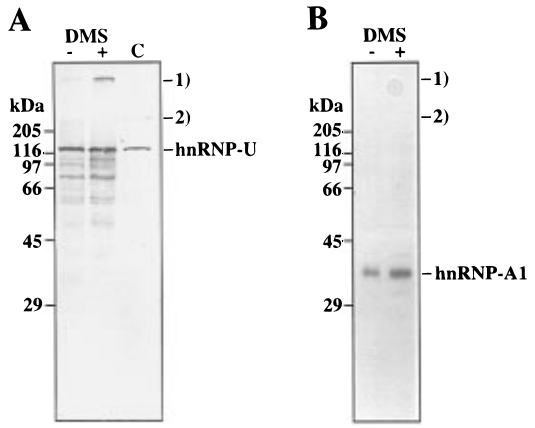

FIGURE 6: DMS cross-linking demonstrates direct DNA binding of hnRNP-U. Purified DNA/protein complexes from a second CsCl gradient were treated with DMS according to the procedure of Bavykin et al. (1993). After reduction with sodium borohydride, aliquots were boiled in SDS−PAGE sample buffer to cleave the methylene bonds introduced by formaldehyde fixation and separated on a 12% polyacrylamide gel (panel A, lane DMS +) along with a sample processed in the same way but without DMS (panel A, lane DMS −) and native, purified hnRNP-U (panel A, lane C). Note that in the sample exposed to DMS, but not in the controls, parts of hnRNP-U are trapped in large complexes unable to enter the stacking gel. The bottom of the well (−1) and the border between stacking and separation gel (−2) is indicated. In panel B, aliquots of the same experiment were used to detect hnRNP-A1 as a control protein not cross-linked to DNA.

one cut is introduced every 300−400 amino acids (30−40 kDa). Complexes treated in this way were then resolved on a (third) cesium chloride gradient and analyzed by Coomassie staining and western blotting of the fractionated gradient (Figure 7). Note that the overall buoyant density of the complexes does not significantly change by this treatment. This most probably reflects the fact that the histones are not grossly degraded by the treatment chosen. By western blotting we find that, in addition to a hardly detectable amount of undegraded hnRNP-U, a major degradation product of hnRNP-U with an apparent molecular weight of approximately 65 kDa in association with DNA/protein complexes (Figure 7C). Experiments using higher protease: protein ratios (1:1000 or 1:750 w/w) gave confirmative results, displaying degradation products of 35 and 20 kDa size, but the total detectable hnRNP-U fragments decreased rapidly with increasing protease concentration (data not shown).

DISCUSSION

To enable rapid dynamic changes, interactions of nucleic acids with proteins in the living cell are usually of a noncovalent nature, mostly on the basis of electrostatic forces. These forces, although strong enough to hold interaction partners together in vivo, are too weak to rule out molecular rearrangements during the purification of native DNA/protein complexes from cells. In this paper we use methods of chemical cross-linking to stabilize DNA/protein binding by introducing covalent bonds between interacting molecules prior to their extraction from the cell. Although covalent cross-links between proteins and DNA can be introduced by a variety of agents, the use of formaldehyde appeared particularly suitable for our purposes. First, formaldehyde can be directly applied to living cells, as it readily penetrates the cellular membranes. Second, the

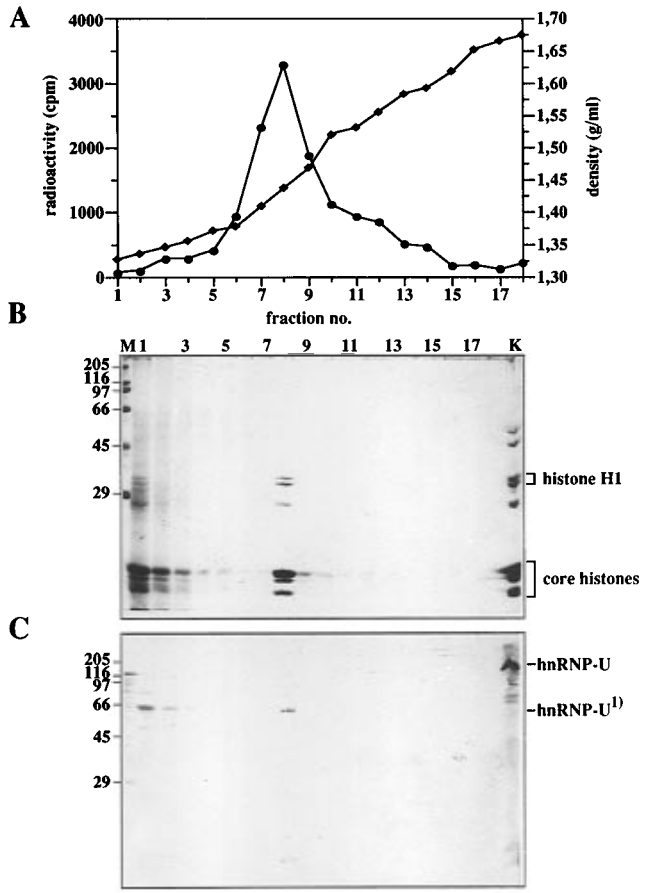

FIGURE 7: Limited proteolysis of DNA/protein complexes. Purified DNA/protein complexes from a second CsCl gradient were treated with proteinase K at a ratio of 1:2000 (proteinase K:total protein in the complexes) for 30 min at 37 °C. Digestion was stopped by the addition of PMSF to a final concentration of 1 mM and CsCl to a density of 1.5 g/mL, and the samples were run on a isopycnic gradient equivalent to the second gradient in the purification (A). Fractions were analyzed for total protein by staining with Coomassie blue (B) or for hnRNP-U by western blotting (C). In panel B, histone H1 and core histones are indicated. In panel C, full-length hnRNP-U (hardly detectable) and the major cleavage product are indicated by hnRNP-U and hnRNP-U[1)], respectively. Note that the complexes sediment in a broader peak in this experiment because of an additional sonication step prior to proteolysis.

formation of cross-links is very fast, virtually "freezing" the situation inside the cell before possible rearrangements can occur. Third, the reaction conditions are easily controlled to cover a range from a limited and specific cross-linking to an extended but unspecific cross-linking. Fourth, and possibly most interestingly in the present context, the covalent bonds introduced by formaldehyde are reversible by boiling in SDS-containing buffers, thus allowing the analysis of cross-linked proteins by gel electrophoresis (Jackson & Chalkley, 1981; Solomon et al., 1988).

Chemically, the "cross-linking" by formaldehyde is the two-step formation of a methylene bond between two primary amines located in amino acid side chains and in the bases of nucleic acids, through a hydroxymethyl intermediate (Fraenckel-Conrat & Olcott, 1948). Due to this reaction mechanism, formaldehyde is a "zero-length" cross-linker that can only cross-link partners close to each other within the length of a single methylene bond. Formaldehyde cross-linking is routinely utilized in the preparation of samples for immunofluorescent microscopy, and numerous experiments have shown that treatment of living cells with

formaldehyde results in the formation of large networks of cross-linked molecules faithfully representing the spatial distribution of these molecules in the cell. Under the conditions of fixation, a complete and unspecific cross-linking is achieved, even including soluble proteins that are not bound to cellular structures in vivo. Obviously, this complete cross-linking is not suitable to investigate the interaction of a given protein with DNA. However, as mentioned above, limited cross-linking can be performed that introduces covalent bonds only between truly interacting molecules.

Several cross-linking procedures with formaldehyde have been described and used previously, e.g., by Jackson and Chalkley (1981) for investigating histone deposition on newly replicated DNA, by Solomon et al. (1988) for analyzing the chromatin structure of the hsp70 gene, or by Orlando and Paro (1993) for mapping polycomb-repressed domains in the *Drosophila* Bithorax complex. In our hands, however, all of these methods suffered from particular shortcomings and appeared unsatisfactory, mostly because they ignored contaminations with non-cross-linked proteins. In initial experiments we devised a modified method that involves treatment of living cells with 1% formaldehyde for 4 min at 37 °C and purification of the resulting complexes over two consecutive CsCl gradients. The complexes prepared in this way are probably closely related to the native state, as their buoyant density is close to that of native chromatin and protein analysis reveals a specific set of cross-linked proteins with histones as major components. Centrifugation on CsCl gradients removes most of the other nuclear proteins that are apparently not cross-linked to DNA, demonstrating the specificity of the procedure we have used. However, when the cross-linking time was extended to 30 min or more, unspecific cross-linking of many additional proteins than the ones we describe could be seen.

Even after short times of cross-linking, a few other proteins are cross-linked to DNA in amounts comparable to that of the histones (Figures 2 and 3), possibly reflecting differences in the cross-linkability of individual proteins. As cross-linking by formaldehyde occurs predominantly via the ϵ-amino group of lysine, proteins exposing several lysines to DNA will presumably be cross-linked more efficiently than those exposing only one or none at all. This will certainly bias the protein composition of the complexes in a way that some DNA-bound proteins might escape detection because they are not cross-linked efficiently, but it cannot lead to a cross-linking if no interaction with DNA (or proteins bound to DNA) is present. Thus, by all criteria tested, the limited cross-linking procedure appears well suited to investigate the DNA binding of specific proteins in vivo.

For our particular case of hnRNP-U, we have performed control experiments regarding a possible cross-linking of the protein to RNA that could influence the interpretation of our data. This appeared necessary as hnRNP-U also binds to RNA in vitro and has been shown to be a component of hnRNP complexes in vivo (Dreyfuss et al., 1984). In our experiments, we have used the same protocol to investigate the binding of hnRNP-U to RNA and to DNA because the chemical basis for both reactions should be identical. However, we found no evidence for the formation of significant amounts of RNA/protein complexes. Rather, bulk RNA is found in the pellet of CsCl gradients where no proteins can be visualized, indicating that RNA is not detectably cross-linked to proteins. A small percentage of RNA appears in the same gradient fractions as DNA/protein complexes, possibly because it has been cross-linked to transcriptionally active chromatin. This amount of RNA is small in comparison to bulk RNA and can be removed from the complexes by RNase digestion with no effect on the buoyant density of the complexes. Thus, the mass of RNA found in DNA/protein complexes constitutes only a minor fraction of these complexes. Summarizing these experiments, we have not been able to demonstrate a binding of hnRNP-U or other proteins to bulk RNA. This obvious discrepancy to the well-established existence of RNA/protein complexes in vivo could be explained in two different ways. First, our cross-linking protocol was optimized for the purification of DNA/protein complexes, so that we cannot rule out that RNA might not be efficiently cross-linked to proteins during our short incubation with formaldehyde. Second, it is conceivable that RNA/protein complexes are formed but are lost during the preparation. The most probable step for such a loss is certainly the extraction step of cross-linked nuclei with 1 M NaCl, necessary for the removal of non-cross-linked contaminants. We have, however, not analyzed the fate of RNA/protein complexes in detail. Nevertheless, all cosedimentation studies were performed with RNase-treated samples to rule out a possible contamination with RNA binding proteins.

By western blotting, hnRNP-U was demonstrated to be present in purified DNA/protein complexes, cosedimenting with bulk DNA and histones on an isopycnic cesium chloride gradient. The amount of hnRNP-U detectable in DNA/protein complexes is approximately 20% of the total cellular hnRNP-U, as judged by western blot quantification. As cross-linking was experimentally limited, and the lengthy procedure of purifying DNA/protein complexes can hardly be considered to be quantitative, we think that this 20% is only a rough estimation, representing the lower limit of the amount of hnRNP-U bound in vivo. However, this percentage corresponds well to results of our earlier studies on subcellular fractionation which had shown that approximately 25% of the total cellular hnRNP-U can be extracted from nuclei by DNase or micrococcal nuclease digestion (Fackelmayer et al., 1994; unpublished observations).

Previous experiments using UV cross-linking had demonstrated a direct binding of hnRNP-U to DNA in vivo (Fackelmayer et al., 1994). In these experiments, 0.1% of the cellular DNA from UV-treated cells, but not from untreated control cells, was immunoprecipitated by anti-hnRNP-U antibodies. Although this result was clear and reproducible, we remained partially unconvinced of its significance because of the low amount of binding observed. In this communication, we have thus used two independent approaches to confirm our conclusion that hnRNP-U is bound directly to DNA. Both approaches, DMS cross-linking and cosedimentation with DNA after limited proteolysis, gave results in favor of a direct binding. Although every single approach has its particular shortcomings, we want to point out that a direct DNA binding has now been established with three independent experimental procedures. In addition, we like to emphasize again (see introduction) that hnRNP-U has a higher affinity for DNA than for RNA in vitro. Also, a method based on the purification of "native" cellular chromatin (Hancock, 1974) without cross-linking demonstrates that hnRNP-U is a component of this chromatin, a

result that fully supports the conclusions drawn from experiments reported in this paper (unpublished).

In summary, our results show that $(4-5) \times 10^5$ molecules of hnRNP-U/nucleus are in direct contact with DNA, strongly indicating that the protein plays a structural role in chromatin organization as previously suggested because of the S/MAR binding specificity. Future experiments will focus on the DNA sites to which hnRNP-U is bound in vivo and on comparing them with the sequence of S/MAR DNA fragments that are the preferred target of the protein in vitro.

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