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Comparison of DNA–Protein Interactions in Intact Nuclei from Avian Liver and Erythrocytes: A Cross-Linking Study

A. Ferraro, L. Cervoni, M. Eufemi, F. Altieri, and C. Turano

Department of Biochemical Sciences and C.N.R. Center of Molecular Biology, University La Sapienza, 00185 Rome, Italy

Abstract DNA–protein cross-linkages were formed in intact nuclei of chicken erythrocytes and liver cells by the action of *cis*-diammine dichloroplatinum (II). Most cross-linked proteins were components of the nuclear matrix, and their heterogeneity reflected the different complexity of liver and erythrocytes matrices, respectively. Some basic proteins, including histones, were also cross-linked, particularly in erythrocyte nuclei. South-Western blotting revealed that a variety of proteins isolated from the cross-linked liver nuclei recognized DNA specifically. In this group of proteins two relatively abundant, acidic, species of 38 and 66 kDa, respectively, might represent novel DNA-binding proteins from the nuclear matrix. In the case of erythrocytes, only the basic proteins showed a DNA-recognition capacity, and among them there were some unidentified species, absent from liver. Lamin B2 was cross-linked but was unable to recognize DNA, and the same was true for other abundant, cross-linked proteins from both types of nuclei. This led to the hypothesis that for some DNA–nuclear matrix interactions the aggregation typical of matrix proteins is essential for the specificity of DNA recognition.

Hybridization analysis of the DNA isolated from the cross-linked complexes showed that SARs (scaffold attachment regions) and telomeric sequences were well represented in the cross-linked fragments, that the cross-linked DNA of liver was partially different from that of erythrocytes and that two defined SAR sequences were found to be present only in the cross-linked DNA. These results are in agreement with the present views on DNA–nuclear matrix interactions, which are usually studied on isolated nuclear matrices or purified proteins. Instead, our results provide experimental evidence obtained directly from intact nuclei.

Key words: DNA–protein cross-linkage, avian nuclei, scaffold attachment regions, *cis*-diamminedichloroplatinum (II), nuclear scaffold

The structural organization of DNA in eukaryotic cells and the regulation of DNA functions are strictly dependent on a multitude of DNA–protein interactions. The prevailing ones are those established between DNA and histones, which are the most abundant nuclear proteins. No less important, however, are the interactions between DNA and nonhistone proteins, involving proteins so multiform as DNA and RNA polymerases, enzymes for nucleic acid and protein modifications, transcription factors, HMG proteins, and nucleoskeletal components. Interest is increasingly attracted nowadays to the nonhistone proteins that provide the attachment sites for the DNA loops and that are thought to be components of the nuclear matrix [Berezney and Coffey, 1974], or nuclear scaffold [Mirkovitch et al., 1984], either at the periphery or in the interior of the nucleus. These interactions are thought to have not only a structural meaning, but also a functional one, since they may contribute to the definition of chromatin domains, their transcriptional regulation and to the proper localization of the replication machinery [for recent reviews, see Getzenberg, 1994; Boulikas, 1995]. Many DNA sequences (SARs) [Gasser and Laemmli, 1986] or MARs [Cockerill and Garrard, 1986]—scaffold or matrix attachment regions, respectively—involved in such interactions have been isolated and a number of proteins interacting with SARs have also been described [Boulikas, 1995]. However, these SAR–protein interactions are far from well characterized.

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Address reprint requests to Carlo Turano, Dipartimento di Scienze Biochimiche, Università La Sapienza, P. Aldo Moro 5, 00185 Roma, Italy.

The binding of SARs, flanking a particular gene, to the nuclear scaffold has often been found related to the actual expression of the gene itself. On the other hand, permanent attachment sites of DNA even in the absence of transcription have also been described (as reviewed by Getzenberg, 1994; Georgiev et al., 1991]. Thus, a general rule for the binding of SARs to the nuclear scaffold in connection with transcription is unknown.

Another uncertainty concerns the proteins involved in the attachment of SARs. About 30 species regarded as participants in such interactions have at present been described, and their capability of a specific recognition of SAR sequences is well established [Boulikas, 1995]. These proteins are usually identified by detecting the specific binding of nuclear matrix proteins to a particular SAR fragment, which in turn has been identified from its binding to a preparation of whole nuclear matrix. The nuclear matrix, however, is not a well-defined subnuclear structure [Cook, 1988; Jack and Eggert, 1992], being rather an operationally defined one, whose composition varies depending on the method used for its preparation, so that even the identification of SARs could, in principle, be uncertain. Furthermore, the potentiality of a certain protein to bind the SARs in vitro does not necessarily demonstrate that the protein actually binds the SARs in vivo.

A logical approach for overcoming these problems could be the use of cross-linking agents, capable of inducing stable bonds between DNA and proteins, and to do so in intact cells or nuclei, thus avoiding the possible artifacts originating from the disruption of nuclei and the preparation of the nuclear matrix. This approach would also be the most appropriate in comparing the protein–SAR interactions taking place in actively transcribing nuclei and in inactive ones, respectively. This would afford an independent approach to check the permanent and transient attachment sites of DNA to the nuclear matrix. We chose, therefore, to use a cross-linking method to analyze and compare the DNA–protein interactions in nuclei prepared from chicken liver and erythrocytes.

We have previously successfully used *cis*-diammine dichloroplatinum (cis-DDP) [Ferraro et al., 1991, 1992], which has been shown by a number of investigators to be a very efficient cross-linking agent [Filipski et al., 1983; Banjar et al., 1984]. Wedrychowski et al. [1986, 1989] and ourselves [Ferraro et al., 1992, 1995b] have demonstrated that many proteins cross-linked in such a way in intact cells or nuclei derive from the nuclear matrix.

From the results of the present investigation, this approach appears to be very convenient to detect and characterize not only the proteins but also the DNA involved in DNA–nuclear matrix interactions within intact nuclei.

MATERIALS AND METHODS

Preparative Methods

Nuclei were prepared from chicken liver according to Blobel and Potter [1966] and from chicken erythrocytes according to Shindo et al. [1980]. The nuclear matrix was prepared according to Berezney and Coffey [1974]. Cross-linking by *cis*-DDP and isolation of the cross-linked proteins by means of hydroxyapatite were performed according to Ferraro et al. [1991]. Usually 50–80 μg of cross-linked proteins per mg of total DNA were obtained. The DNA from the cross-linked complexes was isolated by the use of a gel filtration column, followed by filtration on nitrocellulose membrane [Ferraro et al., 1995a]. This procedure also provided alternative methods to isolate the cross-linked proteins. Briefly, cross-linked nuclei were dissolved in 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 0.5% sodium dodecyl sulfate (SDS), sonicated in ice to give DNA fragments of 800–1,200-base pair (bp), and centrifuged. The clear supernatant was passed through a Sephacryl-HR400 (Pharmacia) column, equilibrated and eluted with the same SDS–Tris buffer. The first eluted fractions, containing large unspecific protein–DNA aggregates, had a relatively low DNA–protein ratio and were discarded. The following fractions, containing most of the DNA, showed a constant DNA–protein ratio and were followed by fractions containing essentially free proteins. The fractions with a constant DNA–protein ratio were precipitated with ethanol. The precipitate was solubilized in 10 mM Tris–HCl buffer, pH 7.4, containing 2 M guanidine HCl, and passed through a nitrocellulose membrane, which was washed first with 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 150 mM NaCl and then with the same solution without NaCl. To dissociate the cross-linked complexes the membrane was treated twice for 1 h at 37°C with 1 M thiourea in 10 mM Tris–HCl

buffer, pH 7.4, plus 1 mM EDTA, and was then washed with the same solution. The DNA eluted in this way amounted to 5–10% of total DNA. To obtain the cross-linked proteins, the nitrocellulose membrane, after the removal of DNA, was fragmented and treated overnight at 37°C in 10 mM Tris–HCl buffer containing 0.25% SDS. Alternatively, the proteins were obtained from the cross-linked complexes eluted from the Sephacryl column, precipitated with ethanol, and digested with nucleases [Ferraro et al., 1995]. The proteins obtained by these methods, analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), showed no significant qualitative differences from those prepared by the hydroxyapatite method. SAR fragments were prepared from liver and from erythrocyte nuclei following the procedure of Mirkovitch et al. [1984].

Analytical Methods

Mono-dimensional electrophoresis was performed according to Laemmli [1970] on 10% gels. Two-dimensional electrophoresis was usually performed according to O'Farrell [1975] with a 10% gel in SDS in the second dimension, while for the analysis of histones and HMG proteins was performed according to Sinclair and Rickwood [1981] in acetic acid/urea in the first dimension, and in SDS in the second dimension on a 15% gel. The electrophoresis gels were stained with Coomassie Blue. For Western blotting, the procedure of Towbin et al. [1976] was followed, and for South-Western blotting that of Bowen et al. [1980], modified according to Du Bois et al. [1990] for the renaturation of proteins after the electrotransfer. The probe for the South-Western experiments was DNA labeled by photodigoxigenin (Boehringer) or by nick translation, using digoxigenin-dUTP (Boehringer) [Muhlegger et al., 1990]. The DNA isolated from the cross-linked complexes was used for this labeling. An extensive purification of DNA was considered unnecessary, because even if small contaminations of residual DNA–protein complexes were present, they would not have affected the South-Western blots. In fact, these complexes are extremely insoluble in the absence of denaturing agents, so they would not have been present in the final solution used for the overlaying of the South-Western blots. Furthermore, the proteins would have been denatured by the ethanol precipitation to which the probe is subjected during the labeling procedure. Competitor DNA was from *Escherichia coli* (Sigma), sonicated to 800–1,200 bp. Comparisons of the two-dimensional electrophoretic separations were performed with a BioRad 620 videodensitometer and the 2D-Analyst II program.

Dot-blot hybridization of DNA was performed on nitrocellulose membranes in a hybridization solution containing 5× SSC, 50 mM Na-phosphate buffer, pH 6.5, 0.2% SDS, 5× Denhardt's solution and 100 μg/ml of E. coli DNA, at 67°C with DNA probes and at 45°C with oligonucleotides probes, for 16 h. The DNA probes were biotinylated with photobiotin [Forster et al., 1985], while the oligonucleotides (Genosys) were biotinylated by the manufacturer. The quantitative evaluation of the hybridization was performed by the use of a BioMed software.

For the Western blots, the antibody against actin was from Sigma (A 2172), and the secondary antibody (Sigma) was linked to peroxidase. Antibodies against lamins B were elicited in rabbits by injection of peptides, which were synthesized and conjugated with keyhole limpet hemocyanin by Tana Laboratories (TX). The peptides were SSRVTVSRASSSRS for lamin B1 [Peter et al., 1989], and SGSGTSGIGTGSIS for lamin B2 [Vorburger et al., 1989]. The sera from immunized rabbits were used as such, and the secondary antibody (Sigma, St. Louis, MO) was conjugated with alkaline phosphatase.

RESULTS

Analysis of the Proteins Cross-linked to DNA

The proteins complexed with DNA after treatment of intact nuclei from chicken liver or erythrocytes with *cis*-DDP were isolated and analyzed by mono- and two-dimensional gel electrophoresis, as shown in Figures 1 and 2. We have previously demonstrated that the majority of cross-linked protein species from chicken liver nuclei are found also in nuclear matrix preparations from the same tissue [Ferraro et al., 1992, 1995b]. It is important to notice that we have deliberately chosen a relatively low-sensitivity staining method (Coomassie Blue stain) for the electrophoresis gels, in order to detect only the major cross-linked protein species, among which it was plausible to find mainly structural components, such as the nuclear matrix proteins.

Comparison of Figures 2B and 3 shows that also the matrix proteins from erythrocyte nuclei were well represented among the cross-linked ones, which, however, had some low molecular mass components showing up in the mono-

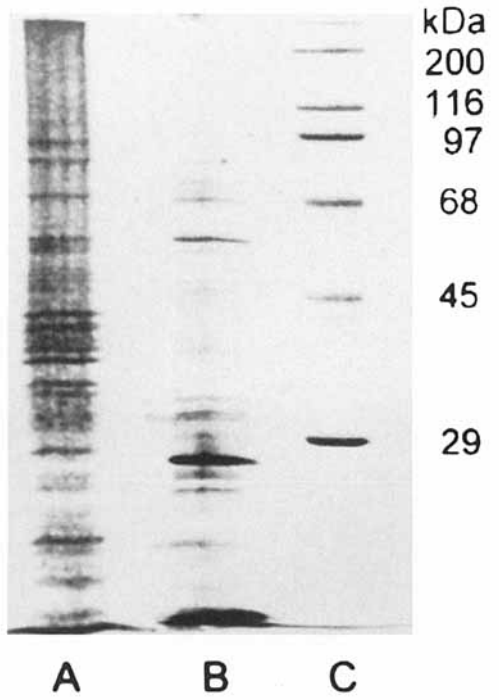

Fig. 1. SDS–PAGE of the proteins isolated from the cross-linked complexes formed in liver nuclei **(A)** and in erythrocyte nuclei **(B)**. Molecular mass markers **(C)**.

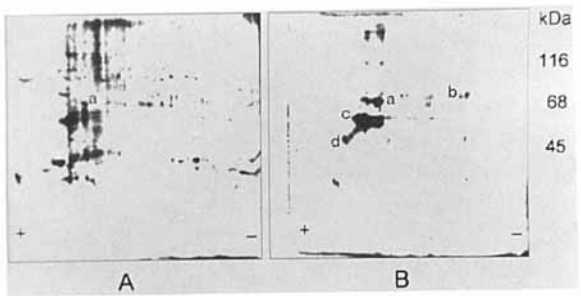

Fig. 2. Two-dimensional gel electrophoresis [O'Farrell, 1975] of the proteins isolated from the cross-linked complexes formed in liver nuclei **(A)** and in erythrocyte nuclei **(B)**. Spots **a–d** indicate the proteins found also in the nuclear matrix from erythrocytes (Fig. 3). Spot a correspond to lamin B2, identified from Western blots (Fig. 5).

dimensional electrophoresis (Fig. 1B), but not in the two-dimensional one. This suggested that these cross-linked species were too basic to enter the IEF gel. Moreover, one of these proteins from erythrocytes (Fig. 1B), similar to that present in smaller amount in liver (Fig. 1A), migrated in the 30-kDa region, showing the abnormal mobility typical of histone H1 in SDS–PAGE.

A two-dimensional electrophoretic separation with a urea-acetic acid run in the first dimension and a SDS-run on a 15% acrylamide gel in the

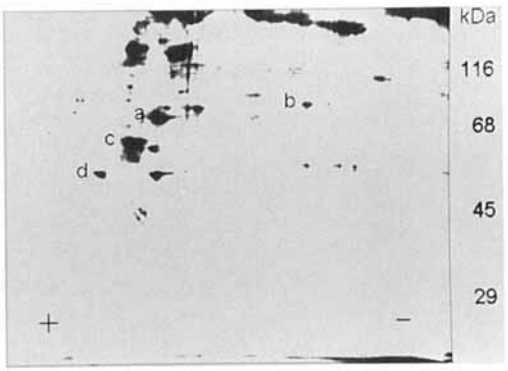

Fig. 3. Two-dimensional gel electrophoresis [O'Farrell, 1975] of the proteins from the nuclear matrix of erythrocytes. Spots **a–d** indicate the proteins cross-linked to DNA by *cis*-DDP (Fig. 2B).

second [Sinclair and Rickwood, 1981] demonstrated that histone H5, trace amounts of H1, and the core histones were present among the cross-linked proteins from erythrocytes (Fig. 4). The identification of the various histones was aided by running standard histone samples alone and also by running pure histone H1 added to the cross-linked proteins (data not shown). The core histones appeared only in the 15% SDS gels (Fig. 4) and not in the 10% gels (Fig. 1), where they run with the front. The core histones and histone H1 in a very low amount were also found among the cross-linked proteins from liver. Beside histone H5, other low molecular mass, basic proteins, as yet unidentified, were present among the cross-linked species from erythrocytes, but not from liver (Fig. 4). Spots which might correspond to HMG proteins 1 and 2 were present in the patterns from liver and from erythrocytes, but they could not be identified with certainty.

The presence of histones was unexpected, since they have been reported not to be subjected to the action of *cis*-DDP [Filipski et al., 1983]. However, an inspection of the mono-dimensional electrophoresis reveals that the amount of cross-linked histones is less than that of non-histone proteins, although histones are much more abundant than any other nuclear protein. Therefore, the appearance of histones among the cross-linked species is the result of a cross-linking reaction occurring with a very low yield.

Western blots of the cross-linked proteins were performed using antibodies against lamin B1, lamin B2 and actin. Lamin B2 appeared to have been cross-linked to DNA both in liver and erythrocyte nuclei, and actin only in liver nuclei (Fig.

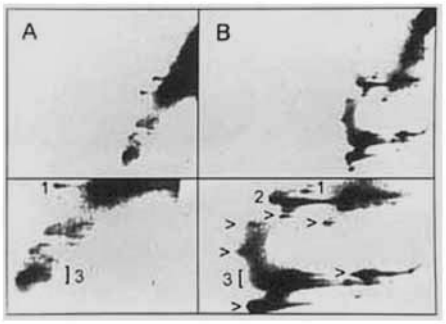

Fig. 4. Two-dimensional gel electrophoresis in acetic acid–urea/SDS [Sinclair and Rickwood, 1981] of the proteins isolated from the cross-linked complexes formed in liver nuclei (**A**) and in erythrocyte nuclei (**B**). Lower panels show an enlarged view of the regions of interest. 1: histone H1. 2: histone H5. 3: core histones. *Arrowheads,* spots present only in B.

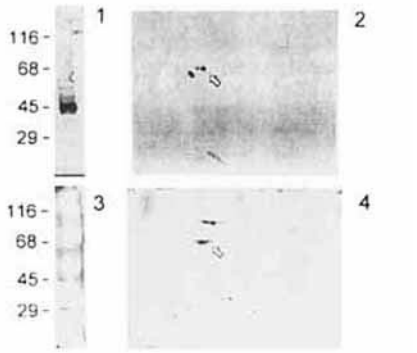

Fig. 5. Western blots of the proteins isolated from the cross-linked complexes formed in liver nuclei (1 and 2) and in erythrocyte nuclei (3 and 4). Anti-actin antibodies were used for the mono-dimensional electrophoresis (1 and 3) and anti-lamin B2 for the two-dimensional electrophoresis (2 and 4). *Arrows,* lamin B2. Anti-lamin B2 revealed also a degradation product of the lamin as shown in panel 2. In panel 4 an unspecific reaction was produced by an unidentified high-molecular-mass component.

5). The antibodies against lamin B1 gave exactly the same pattern as those against lamin B2 (not shown). This could be expected if lamin B1 did not undergo a significant cross-linking to DNA, and if its antibodies were cross-reactive against lamin B2. In fact, while the antibodies against lamin B2 had been elicited against a peptide present only in this protein, the antibodies against lamin B1 were produced using a tetradecapeptide that, with two substitutions, was present also in lamin B2 (see Materials and Methods). This can easily explain the cross-reactivity.

DNA–Protein Recognition

Binding of DNA to the proteins isolated from the cross-linked complexes was studied by South-Western blotting. For this purpose, the DNA from the cross-linked complexes was isolated, labeled, and used to assay its binding to the proteins isolated from the complexes. The patterns of the DNA-binding proteins from cross-linked liver and erythrocyte nuclei are shown in Figure 6. A pattern similar to that in lane A, Figure 6, was obtained when cross-linked proteins from liver were tested with labeled SAR fragments from the same tissue (data not shown).

The proteins binding the labeled DNA in the presence of a 200-fold excess of competitor DNA should possess a certain degree of recognition specificity for DNA. It appears quite clearly that proteins with such property were much more heterogeneous in liver than in erythrocyte, where

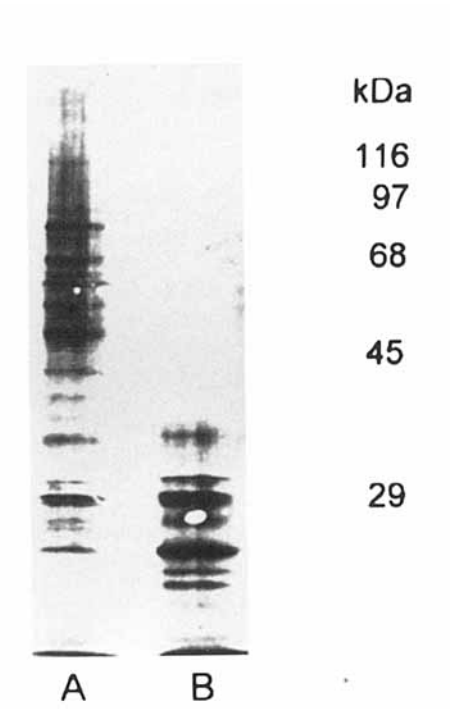

Fig. 6. South-Western blots of the proteins isolated from the cross-linked complexes formed in liver nuclei (**A**) and in erythrocyte nuclei (**B**). The proteins, transferred to membranes and renatured, were probed with labeled DNA isolated from complexes formed in liver nuclei (A) or in erythrocyte nuclei (B), in the presence of a 200-fold excess of *E. coli* DNA.

they were represented only by few low-molecular-mass species. In particular, no binding of DNA could be detected to lamin B2 in erythrocytes, while the binding of DNA to the same protein from liver could not be ruled out. However, a two-dimensional South-Western blot showed unequivocally that lamin B2, also in liver, did not recognize the DNA (Fig. 7). In fact, most DNA-binding proteins were not represented by the most abundant proteins isolated from the cross-linked complexes, as shown by the superposition of the Coomassie-stained gel and the South Western-blot. The only relatively abundant cross-linked species recognizing DNA were two very acidic proteins with molecular masses of 38 and 66 kDa, respectively, a protein of 51 kDa, one in the region of 70 kDa, clearly different from lamin B and probably to be identified with lamin A and/or C, and a 40-kDa protein, with an apparent isoelectric point in the 7.5 to 8 region (Fig. 7).

When two-dimensional South-Western blots of the cross-linked proteins from erythrocytes were performed, no labeled DNA (prepared from the cross-linked complexes) was bound in the presence of competitor (data not shown). This confirmed that lamin B2 did not recognize DNA and demonstrated that the cross-linked proteins from erythrocyte nuclei that were able to bind DNA with specificity of recognition were represented only by the low molecular mass, basic species, not appearing in the two-dimensional electrophoresis gel.

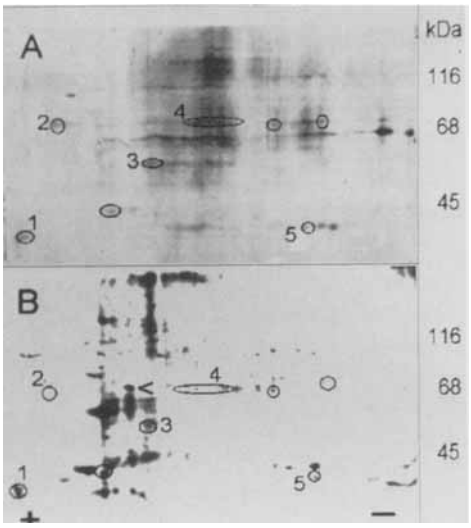

Fig. 7. Two-dimensional electrophoresis [O'Farrell, 1975] of the proteins isolated from the cross-linked complexes formed in liver nuclei. **A:** South-Western blot probed with labeled DNA isolated from the same complexes, in the presence of a 200-fold excess of E. coli DNA. **B:** Coomassie Blue-stained gel (same as that shown in Fig. 2A). The coincident spots of patterns A and B, identified by a computerized comparison, are circled. Numbers 1 to 5 indicate the proteins mentioned in the text, i.e., the 38-, 66-, 51-, 68/70-, and 40-kDa proteins, respectively. *Arrowhead,* lamin B2.

Characterization of DNA from Cross-linked Complexes

The DNA isolated from the cross-linked complexes formed in liver nuclei was isolated, labeled, and used as a probe for hybridization with DNA isolated from cross-linked complexes, with DNA that escaped cross-linking and with SAR fragments, all prepared either from liver or erythrocyte nuclei. As shown in Figure 8, the probe hybridized with all six targets. No hybridization signal was given by 100 ng of target *E. coli* DNA in the same experimental conditions. A lower signal was given by the non-cross-linked DNA from liver than by the cross-linked DNA from the same nuclei (with a 0.5 ratio determined by densitometry). In the case of erythrocytes, a weaker signal was given instead by the cross-linked DNA than by the non-cross-linked DNA (with a 0.3 ratio). Thus, only a fraction of the DNA cross-linked in liver nuclei was also cross-linked in erythrocyte nuclei. SAR fragments from erythrocytes gave, as expected, a lower hybridization signal than produced by those from liver nuclei (with a 0.7 ratio).

Cross-linked and non-cross-linked DNA fragments were also probed with three synthetic oligonucleotides (Fig. 9), one being part of a

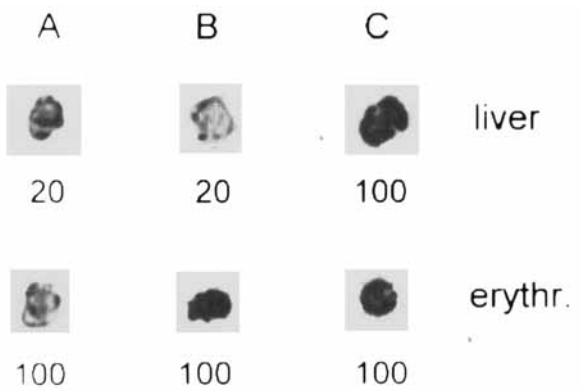

Fig. 8. Dot-blot hybridization analysis of DNA isolated from the cross-linked complexes (**A**), of DNA that escaped cross-linking (**B**), and of SAR fragments (**C**) from nuclei of liver (upper spots) and erythrocytes (lower spots). The probe was DNA isolated from the cross-linked complexes formed in liver nuclei. The numbers indicate the amount of target DNA in nanograms (ng).

MAR sequence located at the 5′ end of the α-globin gene domain [Kolandadze et al., 1990]. Another oligonucleotide was part of the histone genes [Wang et al., 1985], and was located between histones H3 and H2A coding regions, in a segment characterized by many features typical of MAR sequences: an ARS-like fragment [Razin et al., 1986; Gasser et al., 1989], A/T-rich stretches [Gasser and Laemmli, 1986], and an ATATTT sequence [Cockerill and Garrard, 1986]. The third oligonucleotide was a telomeric sequence [Muyldermans et al., 1994].

The first two probes hybridized preferentially, or exclusively, with the DNA prepared from the cross-linked complexes, both from liver and from erythrocyte nuclei. The telomeric probes hybridized with all targets, but preferentially with the cross-linked DNA in the case of erythrocytes, and with the non-crosslinked DNA in the case of liver (Fig. 10). Target DNA from *E. coli*, probed with the three oligonucleotides, produced hybridization signals which were almost indiscernible from the background (data not shown).

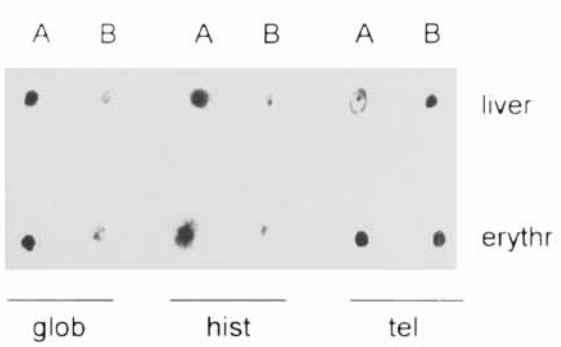

Fig. 10. Dot-blot hybridization analysis of DNA isolated from the cross-linked complexes **(A)** and of DNA that escaped cross-linking **(B)** from liver (upper spots) and erythrocytes (lower spots). The probes were those described in Fig. 9, i.e., oligonucleotides 1 (glob), 2 (hist), and 3 (tel). The amount of target DNA was always 100 ng. The time for color development of the biotinylated probes was much longer for oligonucleotides 1 and 2 than for 3, as expected from their different abundance in genomic DNA.

DISCUSSION

The DNA–protein cross-linking by *cis*-DDP has already been exploited by various authors [Filipski et al., 1983; Banjar et al., 1984; Wedrychowski et al., 1986, 1989]; its favorable characteristics, such as the preferential involvement of nonhistone proteins and its reversibility, have been described [Filipski et al., 1983]. On the other hand, one possible danger of the use of this reagent is the formation of unspecific aggregates of nuclear proteins and nucleic acids, which might even include nearly all the nonhistone nuclear proteins [Filipski et al., 1983], irrespective of their proximity to DNA in the native nucleus. We have overcome this problem by employing new methods to isolate the proteins from the cross-linked complexes [Ferraro et al., 1991, 1992]. In particular, the use of a gel filtration column to fractionate the reaction products and to remove any large aggregate formed ensured that only the specific DNA–protein complexes were analyzed [Ferraro et al., 1995a]. Furthermore, the methods used allowed isolation of either the proteins or the DNA involved in the complexes.

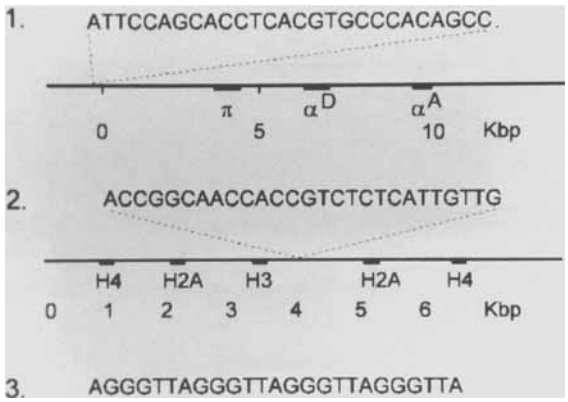

Fig. 9. Probes used for hybridization analysis. **1:** Sequence present at the 5′-end of the chicken α-globin gene domain [Kalandadze et al., 1990]. **2:** Sequence present in the chicken histones genes region [Wang et al., 1985]. **3:** Chicken telomeric sequence [Muyldermans et al., 1994].

The results obtained so far by us with the cross-linking method are compatible with the present views on DNA–matrix interactions. Even if limited, these results illustrate the potentiality of this approach. At least some of the previous findings [Getzenberg et al., 1994; Boulikas, 1995], which concerned the DNA–matrix interactions and which were obtained after the disruption of the nuclei, have been demonstrated to be valid also in the intact nuclei, i.e., in a situation more closely resembling the in vivo conditions. In fact, the cross-linked DNA fragments, which we have shown to be complexed mainly with matrix proteins, should be considered representative of MARs or SARs. The hybridization experiments shown in Fig. 8 demonstrated that this is indeed the case, because the cross-linked DNA from liver nuclei hybridized well with SAR fragments, prepared from liver by conventional methods.

The hybridization experiments showed also that the cross-linked DNA fragments from liver nuclei were present in lower amounts in the cross-linked DNA and SARs from erythrocyte nuclei. This finding is in agreement with the existence of some DNA sequences permanently attached to the matrix, i.e. irrespective of type of tissue, and other sequences attached only in tissues where nearby genes are transcribed [Getzenberg, 1994; Georgiev et al., 1991].

We could also demonstrate that a MAR in the 5′-flanking region of the α-globin gene, previously described as a permanent site of attachment to the matrix [Kalandadze et al., 1990], was present predominantly in the cross-linked DNA fragments, both in liver and in erythrocyte nuclei. The same was found to be true for a sequence in the histones genes region, which has all the characteristics expected for a MAR sequence.

Finally, lamin B2 and actin (in liver) have been identified among the relatively abundant cross-linked protein species. Both proteins have been implicated in the anchoring of DNA to the nuclear matrix [Luderus et al., 1992; Ivanchenko and Avramova, 1992]. Lamins have also been previously cross-linked to DNA in Novikoff [Wedrychowski et al., 1986] and Ehrlich ascites cells [Christova et al., 1989].

Cross-linking by *cis*-DDP allowed also to compare the main proteins involved in the DNA-protein interactions in liver and erythrocyte nuclei, respectively. As expected from the complexity of the nuclear matrix in liver and from the lack of internal matrix in erythrocytes [Lafond and Woodcock, 1983], a much simpler pattern of cross-linked proteins was found in erythrocyte nuclei. It appears legitimate and in agreement with the present views on the function of MAR–matrix interactions to correlate the great variety of protein species cross-linked to DNA in liver nuclei with the high transcriptional activity of the liver cells and their potentiality to replicate, properties missing in mature erythrocytes. In this respect it is interesting to note that evidence has been presented [Egly et al., 1984; Scheer et al., 1984] showing that actin, which we found cross-linked in liver but not in erythrocytes, might be involved in transcription.

Among the cross-linked proteins, histone H5 (in erythrocytes) and the core histones were also identified. As mentioned before, the presence of histones was unexpected, but a rough estimate of their amount led to the conclusion that they had been cross-linked in a very small fraction of the total. The fact that histone H1 has not been cross-linked can be explained by the fact that it is the only histone species containing no histidines, cysteines, or methionines, which are probably the residues involved in the cross-linking reaction [Sherman and Lippard, 1987]. This also explains the presence of histone H5 among the cross-linked proteins from erythrocytes, because this histone, a variant of H1, is found only in erythrocytes and contains three histidine residues.

The patterns of cross-linked proteins from liver and erythrocytes recognizing DNA, as shown by South-Western blots, were also very different, since DNA was bound by a variety of acidic and neutral proteins from liver, but in the case of erythrocytes only by the low-molecular-mass basic proteins.

Surprisingly, however, when the proteins extracted from the cross-linked liver nuclei were analyzed, only a few of the DNA-recognizing proteins corresponded with some major cross-linked species (Fig. 7). The others were clearly present in small amounts, so as to be scarcely detectable in the Coomassie Blue-stained gel. It might be thought that they were minor components of the nuclear matrix, or more probably some soluble nucleoplasmic species. In both cases they might be represented by transcription factors, which have shown to be present not only in the soluble nucleoplasmic fraction, but in the nuclear matrix as well [van Wijnen et al., 1993].

The fact that many abundant cross-linked proteins did not recognize DNA can be explained by their failure to renaturate after SDS–PAGE. It is also possible that these proteins are located in the nucleus within the cross-linking distance from DNA, without being actually bound to it. However, it is worthwhile considering the behavior of lamin B2 and comparing it with what was observed by other investigators. Lamin B2, a relatively abundant component of the cross-linked proteins both in liver and in erythrocyte nuclei, did not recognize DNA, as it appeared from the two-dimensional South-Western blots either from liver or from erythrocytes. Thus, although the cross-linking data demonstrated that DNA in liver and in erythrocytes nuclei is positioned in close proximity to lamin B2, this protein did not show any significant affinity for DNA. This result is in agreement with previous observations [Ivanchenko and Avramova, 1992;

Sperry et al., 1989; Avramova and Paneva, 1992], but it is in contrast with the conclusions of a detailed study by Luderus et al. [1992] that lamins B1 and B2 from rat liver recognize and bind MAR fragments. However, in the South-Western experiments of the last authors only a 10-fold excess of competitor DNA was used. The same authors described a stronger and more specific binding of MARs to nuclear shells, containing complexes of lamins A, B, and C [Luderus et al., 1992], or even to purified lamins B or A/C when the binding of MARs was tested on aggregates formed in solution from native lamins [Luderus et al., 1994]. Thus, the failure of lamin B to recognize DNA in our South-Western experiments might derive from the fact that a proper aggregation process could not occur on the nitrocellulose membrane, where also the right aggregate of lamins A, B and C could not take place.

Therefore, to explain the observation that many cross-linked proteins did not recognize DNA, the hypothesis should be considered that some interactions of DNA with the nuclear matrix take place through multimolecular complexes rather than through single protein molecules or species. While the isolated components of the complex might display a low affinity for DNA, the complex might provide the specificity and the high affinity that have been observed by the in vitro study of MARs or SARs with the isolated nuclear matrix. This would be a situation similar to that of some known multimeric complexes of proteins that have a low-sequence specificity for DNA, but recognize particular conformational features of the double helix, leading to a high-affinity interaction [reviewed by Serrano et al., 1993].

This hypothesis does not exclude the possibility that at least some of the matrix proteins have the individual capability of recognizing and binding DNA. This should be the case, for example, of ARBP [von Kries et al., 1991], of p120 [Tsutsui et al., 1993], of SATB1 [Dickinson et al., 1992], or of other proteins that have been recently identified [reviewed by Boulikas, 1995].

Also in our experiments proteins with this property have been detected. Among the proteins isolated from the cross-linked complexes in liver nuclei, relatively abundant species of 38, 40, 51, 66, and 68–70 kDa have been shown to recognize DNA even in the presence of an excess of competitor DNA. We have previously identified the same cross-linked proteins as components of the nuclear matrix [Ferraro et al., 1995b]. The DNA-binding proteins of 68–70 kDa could be lamins A/C, in agreement with what was observed by Hakes and Berezney [1991]. It should be noticed that the 38- and 66-kDa proteins are quite acidic, and this supports the view that they bind DNA specifically, rather than simply by an electrostatic effect.

Regarding the interactions of DNA with the matrix in erythrocytes, identified by the cross-linking experiments, our results indicate that they take place mainly with lamin B2 and with acidic proteins in the 50- to 60-kDa region. Other interactions involve basic low-molecular-mass proteins, including some species, as yet unidentified, which are absent from the cross-linked proteins of liver. HMG proteins 1 and/or 2 might be present among the cross-linked species from liver and erythrocytes (Fig. 4), and they could contribute to the binding of DNA. In fact they have already been shown to become cross-linked to DNA by *cis*-DDP [Scovell et al., 1987].

The basic cross-linked proteins from erythrocytes show a preferential binding to cross-linked DNA, as indicated by the South Western blots (Fig. 6). This was expected for histone H5, which being similar to histone H1 is likely to share with it the affinity for A/T-rich sequences [Izaurralde et al., 1989].

These data do not yet provide a clear picture of the structure existing at the base of the DNA loops in erythrocytes. It is possible that the attachment takes place mainly or exclusively at the nuclear periphery. It is also possible that, since the multitude of high-affinity SAR-binding proteins found in actively transcribing nuclei are absent in erythrocytes, the SAR sequences, or any A/T-rich region are bound by the basic proteins recognizing these sequences, such as histones H1 and H5, HMG proteins, and perhaps also the basic, unidentified species found among the cross-linked proteins. This hypothesis is in agreement with the model by Käs et al. [1992] of chromatin compaction. It was proposed that in the absence of other proteins binding to A/T tracts, histone H1, or other linker-binding proteins, would bind tightly to these regions and nucleate the assembly of other H1 molecules along the chromatin fiber, which would therefore exist in a compacted, inactive state.

Our results show that DNA-proteins interactions in liver or erythrocyte nuclei differ also for another important feature. From hybridization

experiments it appeared that the telomeric sequences were present predominantly in the cross-linked DNA from erythrocyte nuclei, and in the non-cross-linked DNA from liver nuclei. The enrichment of cross-linked DNA from erythrocytes in telomeric sequences cannot be attributed to the fact that histone H5 was partially cross-linked, since Muyldermans et al. [1994] demonstrated that the ratio of histone H5 to histone H1 is much lower in telomeric than in bulk chromatin.

Telomeres have been shown to be bound to the nuclear matrix [de Lange, 1992], and telomere-binding proteins have been described [Blackburn, 1991]. Our data demonstrate that the interactions of telomeric sequences with non-histone proteins is not constant in all cell types, but appears to prevail in the inactive erythrocyte nuclei, rather than in the transcriptionally active liver nuclei. However, a wider range of cell types and organisms should be examined before this feature is accepted as a generally valid one.

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