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DNA-nuclear matrix interactions analyzed by cross-linking reactions in intact nuclei from avian liver*

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To detect the interactions of DNA with the nuclear matrix proteins, DNA-protein cross-linkages were induced in intact nuclei from chicken liver by the use of *cis*-diammine dichloroplatinum. Methods have been devised for fast purification both of the proteins and of the DNA fragments involved in the cross-linked complexes. By Southern-Western blotting a number of matrix proteins isolated from the complexes have been shown to recognize specifically DNA sequences present in the cross-linked DNA fragments. This experimental approach not only allows to identify the nuclear matrix-DNA interactions existing in the nucleus before its disruption, but also provides a preparation of matrix proteins enriched in those species which are involved in such interactions and which can therefore be detected with high sensitivity.

The cell nucleus is provided with a structural protein meshwork, called nuclear matrix or scaffold, to which large loops of chromatin DNA are anchored [1, 2]. The nuclear matrix has been shown to be involved in a variety of processes, like replication and transcription of DNA [3], and splicing and transport of RNA [4]. Nuclear matrix, or nuclear scaffold, associated regions of DNA (MAR or SAR, respectively) were identified by isolating DNA fragments bound to matrix preparations [1, 2, 5]. A number of nuclear matrix proteins, including topoisomerase II [6], were identified as capable of specific binding to MAR fragments, and have therefore been postulated to be involved in the anchoring of DNA loops to the scaffold. However, there are still many unelucidated problems concerning the nuclear matrix structure; specific protein-MAR interactions found *in vitro* do not necessarily exist *in vivo*, or might exist only in particular phases of the cell cycle or under some particular conditions.

In an attempt at overcoming these difficulties, we found that it was fruitful to perform DNA-protein cross-linkage reactions on intact cells or nuclei by the use of *cis*-diammine dichloroplatinum (*cis*-DDP), in such a way as to stabilize the DNA-protein complexes existing *in vivo* [7]. These could then be isolated and characterized, avoiding the possible artifacts that might otherwise arise during the disruption of cells or nuclei.

By the use of the same reagent, we have also shown that many proteins cross-linked to DNA

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[1]Abbreviation: *cis*-DDP, *cis*-diamminedichloroplatinum

in liver cell nuclei are identical, on the basis of two-dimensional electrophoretic analysis, to proteins found in the internal and peripheral nuclear matrices [8], prepared from the same tissue according to Kaufmann & Shaper [9].

Considering the promising features of *cis*-DDP, we analysed in greater detail the protein-DNA complexes formed in chicken liver nuclei by this reagent, looking for the specificity of DNA recognition of the proteins isolated from the cross-linked complexes. The results obtained indicate that the use of *cis*-DDP provides a valuable approach to identification of the interactions between matrix proteins and DNA existing *in vivo*, and for identification of matrix proteins involved in specific recognition of the MARs.

MATERIALS AND METHODS

Nuclei were isolated from chicken liver according to Blobel & Potter [10]. The proteins of whole nuclear matrix were obtained according to Berezney & Coffey [11]. DNA-protein cross-linking in intact nuclei by *cis*-DDP was performed according to Ferraro *et al.* [7] (1 mM *cis*-DDP, at 37°C for 2 h). All buffers contained 1 mM phenylmethane sulfonylfluoride (Sigma), and during incubation of nuclei with *cis*-DDP and during matrix isolation 1 μM pepstatin and 10 μM amido-phenylmethylsulfonyl fluoride (all from Boehringer) were also added. DNA was isolated from cross-linked complexes by treating cross-linked nuclei with lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.5% SDS) and subjecting them to extensive sonication at 4°C to give an average range of DNA fragments of 0.8–1.2 kb as determined by agarose gel electrophoresis. The suspension was centrifuged for 20 min at 10000 × *g*; 2 ml of the clarified supernatant (180 A_{260} units) was loaded on a Sephacryl 400 column (90 cm × 2.5 cm) and eluted with lysis buffer at 20 ml per hour. The eluted fractions were analyzed for absorbance at 260 and 280 nm and for protein content. The fractions containing DNA (i.e., both free and complexed to proteins) were pooled and subjected to ethanol precipitation. The precipitate was collected and processed in different ways to isolate the cross-linked proteins or the cross-linked DNA, respectively.

For isolation of the complexed proteins the DNA was removed either by digestion with a nuclease by conventional methods, or else, after dissociation of the complexes by incubation with 1.5 M thiourea at 37°C for 1.5 h [12], by selective protein precipitation with SDS-KCl as described by Mirzabekov *et al.* [13].

For isolation of the complexed DNA fragments, the precipitate was dissolved in 2 M guanidine/HCl in 10 mM Tris/HCl buffer, pH 7.4, and passed through a nitrocellulose membrane. The latter was washed with 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA, then with the same solution without NaCl, and finally was treated twice for 1 h at 37°C with 1 M thiourea in 10 mM Tris buffer, pH 7.4, plus 1 mM EDTA. The membrane was then washed with the same solution; the DNA fragments so eluted were dialyzed against 1 mM phosphate buffer, pH 7.4, and labeled as described hereafter.

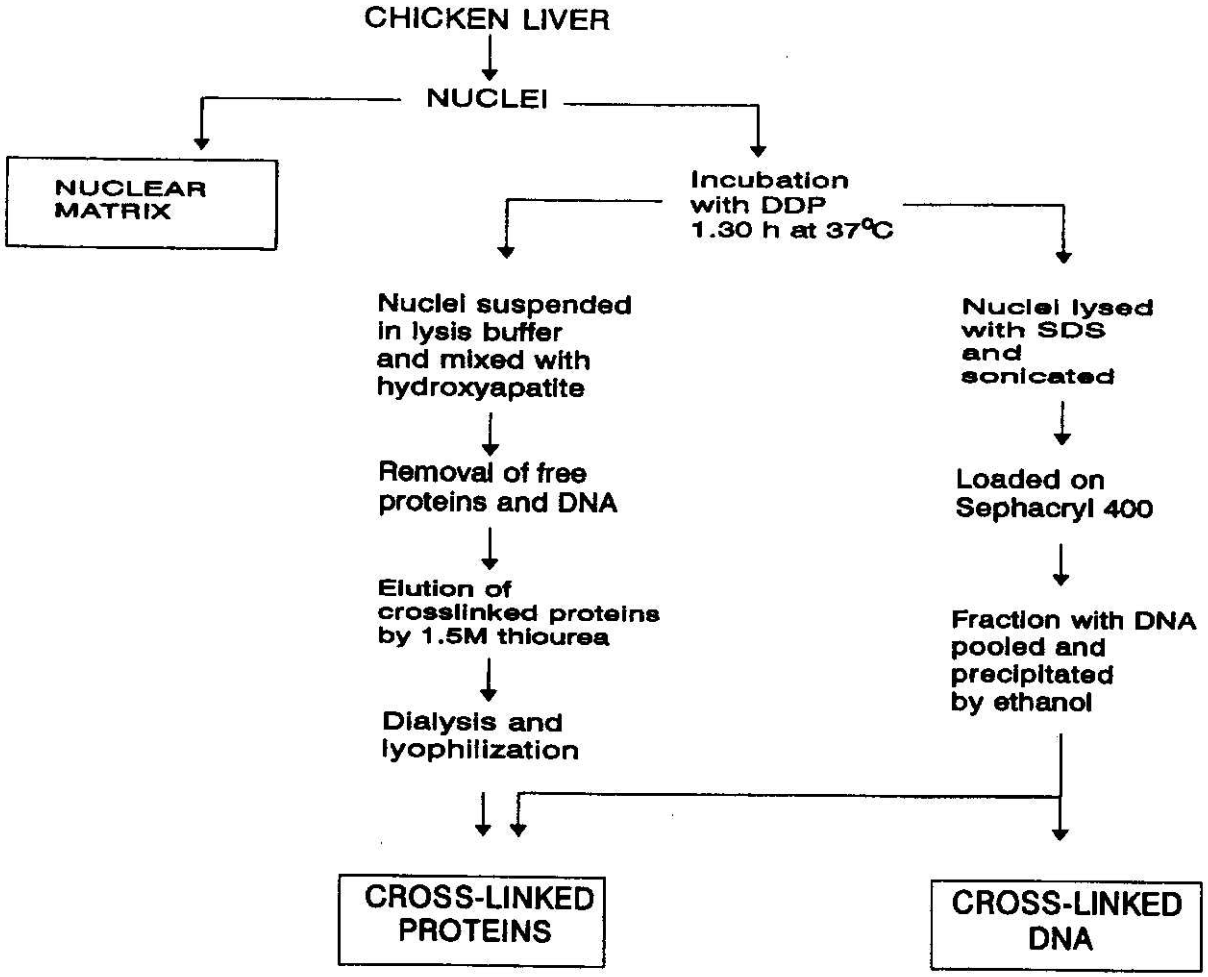
Proteins from cross-linked complexes could also be obtained by a different method using hydroxyapatite [7].

The protein fractions were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis according to Laemmli [14] in 10% polyacrylamide gels. Proteins were stained with Coomassie Blue or electrotransferred to nitrocellulose membranes (Millipore) according to Towbin *et al.* [15]. After electrotransfer the proteins were renatured according to Du Bois *et al.* [16]. The membranes were then incubated overnight in buffer A (10 mM Tris, pH 7.5, 35 mM NaCl) containing 2% bovine serum albumin and washed for 30 min with three changes of buffer A. For detection of DNA-binding proteins the nitrocellulose blots were incubated for 1 h in a small volume of buffer A containing labeled DNA fragments isolated from cross-linked DNA complexes as described above, at the concentration of 0.1 μg/ml, in the absence or presence of 5–20 μg/ml unlabeled *E. coli* competitor DNA. Unbound DNA was removed by washing the blots with three changes of buffer A for 10 min each.

The labeling of DNA fragments was performed by the use of photodigoxigenin (Boehringer) or by nick translation with digoxigenin-11-2′-deoxyuridine-5′-triphosphate (Boehringer), according to Mühlegger *et al.* [17]. Staining was performed by anti-digoxigenin-alkaline phosphatase [18].

RESULTS

Nuclei from chicken liver, treated with *cis*-DDP, were subjected to one of the two procedures shown in Scheme 1. The new Sephacryl gel-filtration method was used both to prepare the cross-linked proteins, or the cross-linked DNA. The elution profile is shown in Fig. 1. Nucleic acid fragments, both free and complexed to proteins, emerged first, as shown by



Scheme 1

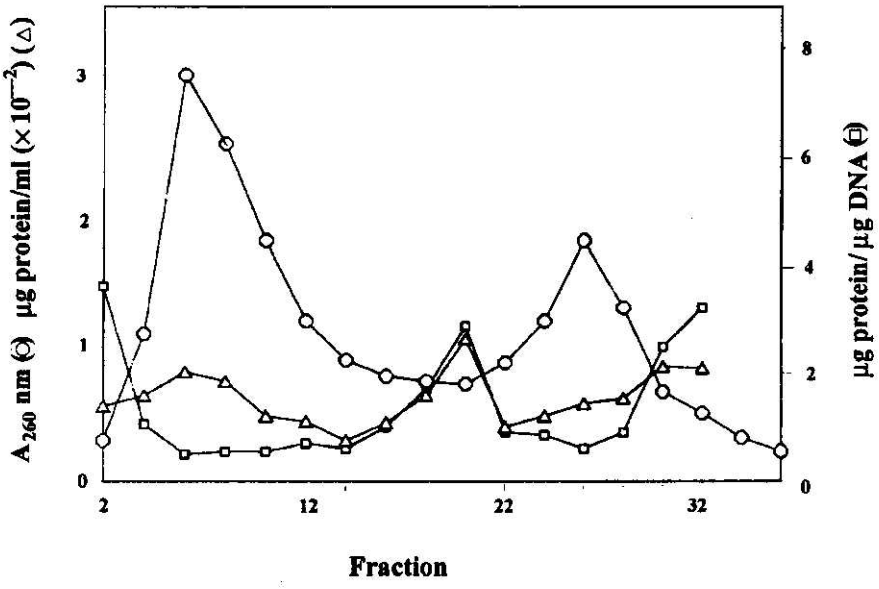


Fig. 1. Elution of an SDS-lysate of cross-linked nuclei from a Sephacryl-400 column.
○, Absorbance at 260 nm; △, protein concentration; □, protein/DNA ratio (µg/µg).

the absorbance peak with a very high A_{260}/A_{280} ratio. A peak containing free proteins followed. Some material eluted in the void volume of the column just before the nucleic acid peak was found to have a particularly low value of the nucleic acid/protein ratio. This could be attributed to some unspecific aggregates, formed and stabilized by the platinum complex. On the other hand, the nucleic acid/protein ratio maintained a constant value over the main nucleic acid peak, indicating a homogeneous distribution of the cross-linked DNA-protein complexes. The fractions corresponding to this material were collected, and served to prepare either the complexed proteins after their dissociation from DNA by thiourea or digestion of DNA, or the complexed DNA, after filtration on nitrocellulose and dissociation from the proteins by thiourea. When the proteins derived from the complexes were analyzed by SDS-PAGE, they gave patterns which were essentially the same as those obtained by the hydroxyapatite procedure (Fig. 2). The differences were mainly quantitative, and probably arose from the fact that two different preparations of nuclei were used. When the cross-linking reaction was performed under the conditions indicated under Materials and Methods, 150 μg of DNA was obtained from cross-linked complexes from 6 mg of nuclei (measured as DNA).

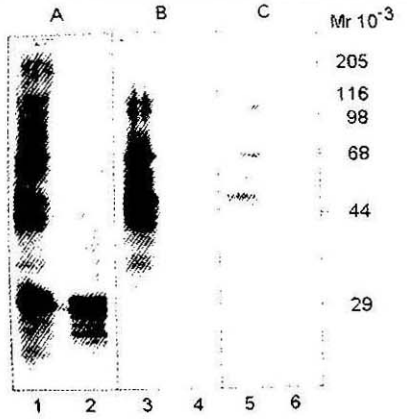


Fig. 3. Southern-Western blots of proteins isolated from cross-linked complexes.
Labeled DNA extracted from cross-linked complexes was used as a probe, in the absence (A), or in the presence of a 50- (B) or 200-fold (C) excess of competitor *E. coli* DNA. Lanes 1, 3 and 5: cross-linked proteins. Lanes 2, 4 and 6: histone H1.

To check whether the proteins and the DNA derived from the complexes were capable of reassociating *in vitro*, a Southern-Western blotting procedure was performed. For this purpose the proteins were fractionated by SDS-PAGE, transferred to nitrocellulose mem-

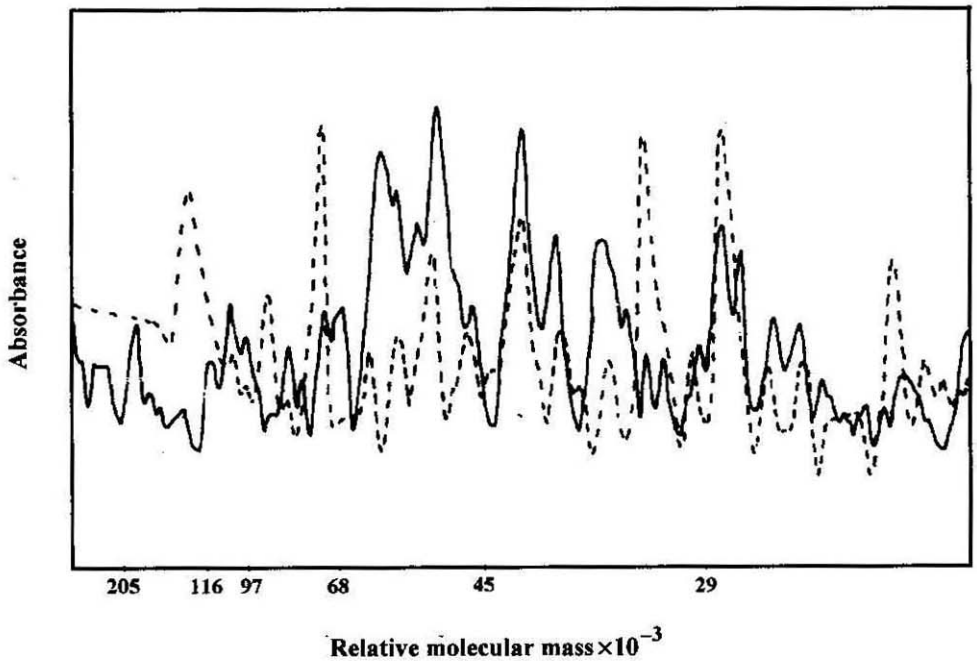


Fig. 2. Densitometric scans of SDS-polyacrylamide gel-electrophoresis patterns of proteins isolated from cross-linked complexes and stained with Coomassie Blue.
—, Isolation by the hydroxyapatite procedure; - - -, isolation by the Sephacryl procedure.

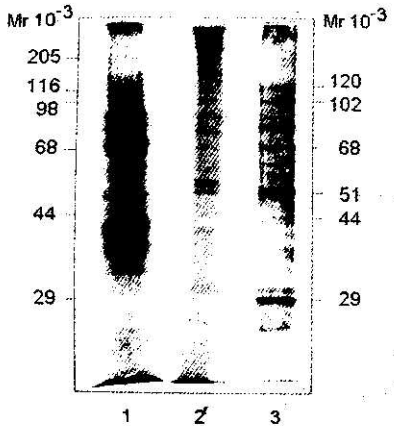


Fig. 4. Electrophoretic patterns of proteins isolated from cross-linked complexes and of nuclear matrix proteins.

Lane 1: cross-linked proteins, stained with Coomassie Blue. Lane 2: nuclear matrix proteins, stained with Coomassie Blue. Lane 3: Southern-Western blot of cross-linked proteins, with labeled DNA from cross-linked complexes as a probe, in the presence of a 200-fold excess of competitor DNA from *E. coli*. Relative molecular mass values of standard proteins are shown at the left side, those of the main protein bands recognizing the labeled DNA at the right side.

branes, renatured and overlayed with labeled DNA derived from the complexes. As shown in Fig. 3, lane 1, DNA did indeed bind to a great number of protein species. The specificity of protein-DNA interactions was verified by the addition of increasing amounts of competitor *E. coli* DNA to the labeled, homologous DNA. As shown in Fig. 3, lanes 3 and 5, the addition of a 50- or 200-fold excess of competitor DNA decreased the binding of labeled DNA to several of the proteins bands, but not to three main species, with relative molecular mass of about 102 000, 68 000, and 51 000, respectively. The behaviour of histone H1, shown in lanes 2, 4 and 6 paralleled, as expected, that of proteins which bound labeled DNA nonspecifically.

Some faint bands of DNA-binding proteins were still visible in lane 5, where proteins derived from cross-linked complexes had run, and competitor DNA was in a 200-fold excess with respect to labeled DNA. Therefore Southern-Western blots were repeated under the same conditions, but with a higher amount of proteins, as shown in Fig. 4, lane 3. The same three major DNA-binding proteins appeared, plus a variety of minor ones.

When patterns of DNA-binding proteins are compared to those obtained from cross-linked proteins and from nuclear matrix proteins, both stained with Coomassie Blue (Fig. 4, lanes 1 and 2), it seems that the main cross-linked protein species, capable of DNA binding, are components of the nuclear matrix.

DISCUSSION

The cross-linking of proteins to DNA in intact cells or nuclei is an efficient method for the identification of protein-nucleic acid interactions prior to disruption of the nuclear structure, avoiding in this way the possible artifacts which could accompany the preparation of subnuclear components. UV irradiation is the method usually employed to induce such cross-linkages. However, the use of heavy metal for this task has been shown to provide distinct advantages [19]. In particular, *cis*-DDP is more effective than UV irradiation in promoting cross-linkages, it does not induce breaks in the DNA strands, it does not act on histones, which could otherwise become the major DNA-complexed proteins, and finally gives DNA-protein cross-linkages which can be readily dissociated by thiourea [12]. However, the originally described method to identify the proteins involved in the complexes was rather time-consuming, requiring 24 h to 48 h of ultracentrifugation [19]. It should be also taken into account that metal complexes might give origin to unspecific macromolecular aggregates when the treated cells or nuclei are broken as an initial step of protein purification. Moreover, it has never been proved that *cis*-DDP makes possible to detect real interactions between DNA and proteins taking place *in vivo* rather than just their proximity. The methods and experiments described in this paper were intended to assess critically the usefulness of *cis*-DDP for identification of these interactions.

The two methods applied here, which use hydroxyapatite or Sephacryl 400, respectively, for separation of free proteins from DNA and DNA-protein complexes, gave essentially the same results in terms of proteins identified as components of the cross-linked complexes, although the principle of the separation and also the dissociating media were completely different. The method based on Sephacryl, in particular, is fast, since it allows to obtain the DNA fractions, containing both free DNA and DNA-protein complexes, in less than two hours. It

also guarantees that any unspecific, high molecular mass aggregates are eliminated, and it allows to isolate not only the protein components of the cross-linked complexes, but also the DNA.

Southern-Western blotting experiments have demonstrated conclusively that most of the proteins isolated from the complexes are capable of binding DNA, indicating that *cis*-DDP does indeed cross-link and stabilize interactions taking place in the intact nucleus, and not that it reveals only a proximity of proteins and nucleic acid. In this respect, therefore, *cis*-DDP is not inferior to a zero-length cross-linker like UV irradiation.

The use of increasing amounts of heterologous competitor DNA in the Southern-Western blots has also revealed that many proteins isolated from the cross-linkages recognize specifically sequences and/or conformations of the interacting DNA. As suggested by Fig. 4, most of these proteins seem to derive from the nuclear matrix, where they might constitute the anchorage sites for DNA loops. If this is the case, the interacting DNA fragments should contain the MAR sequences, which have been previously identified following a different approach. In fact, preliminary experiments have shown that labeled MAR fragments, prepared from chicken liver nuclei according to Mirkovitch *et al.* [20] (not shown), are recognized and bound by the same proteins which bind DNA (derived from cross-linked complexes), as described above.

Although so far the cross-linked proteins which recognize DNA in the Southern-Western blots have neither been identified nor characterized, it can be suggested that the 68000-M_r polypeptide is lamin B, which Ludérus *et al.* [21] have previously described as a MAR-binding protein. A protein with a similar relative molecular mass has been previously detected by Oliński *et al.* [22] among the DNA- *cis*-DDP complexed proteins in Novikoff hepatoma cells.

As a whole, the data reported indicate that the cross-linking reaction involving *cis*-DDP is well suited for identification of protein-DNA interactions taking place in the intact nucleus, and particularly of those occurring at the level of the nuclear matrix. The methods described allow to prepare a subset of nuclear proteins enriched in matrix components with DNA-binding capabilities. Furthermore, the Sephacryl method appears to be particularly suitable for isolation of the purified protein-DNA complex by immunoprecipitation procedures.

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