# Glycoproteins of Chicken Liver Nuclei Cross-Linked to DNA by cis-diamminedichloroplatinum

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**Abstract.** Glycoproteins which participate in DNA-protein cross-links induced by action of *cis*-diamminedichloroplatinum (*cis*-DDP) in intact nuclei of chicken liver were investigated. Digoxigenin-labelled lectins with different sugar specificity were used for detection and characterization of these glycoproteins. Our results showed the presence of glycoproteins bearing high mannose as well as complex type oligosaccharides in chicken liver nuclei. In most cases of complex oligosaccharides, sialic acid residues bound in $\alpha$(2-6) but not in $\alpha$(2-3) linkage were present.

**Key words:** *cis*-diamminedichloroplatinum — Nuclear glycoproteins — Chicken liver — Immunodetection — Digoxigenin-labelled lectins

## Introduction

DNA-protein cross-linking is a widely used method in the study of macromolecular interactions occurring in the nucleus. When it is performed on intact nuclei or on whole, viable cells, it stabilizes these interactions as they occur *in vivo* (Cervoni et al. 1999). It was found out that many nuclear proteins bind to DNA modified by platinum compounds such as *cis*-DDP and *trans*-DDP (Banjar et al. 1984; Ferraro et al. 1991, 1992, 1996; Hughes et al. 1992; Walter et al. 1997; Cervoni et al. 1999). *trans*-DDP easily forms histone-histone and histone-DNA cross-links while *cis*-DDP preferentially cross-links DNA to nonhistone proteins placed in its proximity (Banjar et al. 1984; Cervoni et al. 1999). A few reports demonstrated that many proteins cross-linked to DNA by *cis*-DDP in intact cells or nuclei derived from the nuclear matrix. Ferraro et al. (1992) reported that at least eleven polypeptides cross-linked to DNA in chicken liver cells were found among the proteins of the nuclear matrix. Five of them were found also among cross-linking proteins from ox and pig liver cells. The finding of these highly conserved matrix proteins involved in the DNA binding suggests that they have a unique role in the anchorage of chromatin DNA. Lamin B2 and actin were identified among the relatively abundant cross-linked protein species of chicken liver nuclei. Both proteins were implicated in

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DNA anchoring to the nuclear matrix (Ferraro et al. 1996). Hybridization analysis of DNA isolated from cross-linked complexes showed that SARs (scaffold attachment regions) and telomeric sequences were well represented in the cross-linked fragments (Ferraro et al. 1996).

*cis*-DDP induced DNA intrastrand adducts are specifically recognized also by high mobility group proteins, i.e. HMG1 and HMG2 (Hughes et al. 1992). These chromosomal non-histone proteins are very ubiquitous, highly conserved and abundant. Although HMG1 and HMG2 have been extensively studied their cellular role remains still unclear (Bustin and Reeves 1996). It has been supposed that cross-linking of these proteins to DNA can modify chromatin structure and thus affect the regulation of genome expression.

The aim of the present study was to detect and characterize glycoproteins from chicken liver nuclei which participate in DNA-proteins cross-links induced by action of *cis*-DDP.

## Materials and Methods

Nuclei were isolated from chicken liver by a modified sucrose method as previously described (Krześlak and Lipińska 2001). All solutions for preparation were used at 4℃ and contained 1 mmol/l phenylmethylsulphonyl fluoride (PMSF).

Proteins which participate in DNA-protein cross-links induced by *cis*-DDP were isolated as described by Ferraro et al. (1991). However, as a starting material nuclei were used instead of intact cells. The nuclear pellet was suspended in solution containing 0.25 mol/l sucrose, 5 mmol/l $MgCl_2$, and 0.8 mmol/l $KH_2PO_4$ at pH 6.7. *cis*-DDP was added at a final concentration of 1 mmol/l, and the mixture was incubated with shaking for 2 hours at 37℃. After incubation the nuclei were suspended in: 200 mmol/l potassium phosphate buffer, pH 7.5 containing 5 mol/l urea, 2 mol/l guanidine-HCl, 2 mol/l NaCl, 1 mmol/l PMSF and mixed with hydroxylapatite (1 mg DNA *per* 2 ml hydroxylapatite). After 1 h incubation at 4℃ the suspension was centrifuged for 10 min at 10,000 × *g* and washed several times with the same buffer. Finally the proteins cross-linked to DNA were released from hydroxylapatite by a 2 h incubation in the above mentioned buffer in which urea was substituted by 1 mol/l thiourea. The proteins released by thiourea treatment were dialysed and then lyophilized.

Nuclear protein samples were mixed with 0.9 vol. of solubilizing buffer (20% glycerol, 4% sodium dodecyl sulphate (SDS), 25 mg/l Pyronine Y, 0.125 mol/l Tris-HCl buffer, pH 6.8) and with 0.1 vol. of 2-mercaptoethanol, and then heated in a boiling water bath for 5 min. One-dimensional electrophoresis was performed in slab polyacrylamide gels containing 0.1% SDS and 8% acrylamide (pH 8.8) with 3% stacking gel (pH 6.8) according to Laemmli (1970) at 25 mA/slab gel until the Pyronine Y marker reached the end of the stacking gel, and then at 35 mA/slab in the resolving gel until the marker dye reached the bottom of the gel. The slab gels were stained with Coomassie Brilliant Blue R-250 according to Fairbanks et al. (1971) and with silver method described by Wray et al. (1981). Relative

molecular masses of protein bands were calculated by comparison with the standard proteins: myosin (205 kDa), $\beta$-galactosidase (116 kDa), phosphorylase $b$ (97 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and $\alpha$-lactoalbumin (14.4 kDa).

Proteins separated in SDS-polyacrylamide slab gels were transferred onto Immobilon-P transfer membranes (pore size 0.45 $\mu$m) by electrophoretic blotting in 20% methanol, 192 mmol/l glycine, and 25 mmol/l Tris (pH 8.3) for 15 h at 60 V and 4°C (Towbin et al. 1979).

The detection of lectin-binding glycoproteins immobilized on Immobilon-P sheets was accomplished by the method of Haselbeck et al. (1990). The membranes were treated for 30 min at room temperature with 20 ml 0.5% blocking reagent (w/v) in Tris buffered saline (TBS: 50 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.5), and were washed twice with TBS and once with buffer 1 consisting of TBS, 1 mmol/l $MnCl_2$, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, at pH 7.5 (50 ml each). The membranes were incubated for 1 h at room temperature with the lectin-digoxigenin (DIG) conjugate (GNA, DSA, SNA – 1 mg/l; RCA-120 – 10 mg/l; MAA – 5 mg/l) in 20 ml of buffer 1 (DIG-lectins have been tabulated in Table 1).

**Table 1.** Digoxigenin-labelled lectins used in this study and their sugar specificity

| Lectin | Specificity |
|---|---|
| GNA (*Galanthus nivalis* agglutinin) | Man ($\alpha$ 1-2, 1-3, 1-6) Man |
| RCA-120 (*Ricinus communis* agglutinin) | $\beta$ Gal/$\alpha$ Gal |
| DSA (*Datura stramonium* agglutinin) | Gal ($\beta$ 1-4) Glc*N*Ac |
| SNA (*Sambucus nigra* agglutinin) | SA ($\alpha$ 2-6) Gal |
| MAA (*Maackia amurensis* agglutinin) | SA ($\alpha$ 2-3) Gal |

Then, the membranes were washed three times with 50 ml TBS, and polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (anti-DIG AP, 750 U/ml) were added in a 1 : 1,000 dilution in 20 ml TBS and incubated for 1 h at room temperature. The membranes were again washed three times with 50 ml TBS and the alkaline phosphatase reaction was carried out by incubating the membrane without shaking in 20 ml of the following freshly prepared solution: 75 $\mu$l 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt (BCIP 50 g/l, in dimethylformamide) and 100 $\mu$l 4-nitro blue tetrazolium chloride (NBT 75 g/l, in 70% dimethylformamide) in 20 ml of buffer 2 (100 mmol/l Tris-HCl, 50 mmol/l $MgCl_2$, 100 mmol/l NaCl at pH 9.5). The reaction was completed within a few minutes. The membranes were raised with $H_2O$ to stop the reaction and allow drying.

The protein content was assayed using bovine serum albumin as a standard by the method of Lowry et al. (1951) and the DNA concentration was determined spectrophotometrically.

All reagents used for the detection and characterization of glycoproteins on blots were from Roche (Germany). Bio-gel HTP (hydroxylapatite) was purchased from Bio-Rad Laboratories (Hercules, California, U.S.A.). Other reagents were of the highest purity available and were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). The Immobilon-P transfer membranes were obtained from Millipore Corporation (Bedford, Massachusetts, U.S.A.).

## Results

In our investigation of chicken liver glycoproteins which participate in DNA-protein cross-links induced by action of *cis*-diamminedichloroplatinum (*cis*-DDP) we used as a starting material nuclei instead of whole cells. Nuclei were carefully isolated and checked for integrity and purity by observation with light microscopy. Distinct non-nuclear contaminations were absent in purified nuclear preparations.

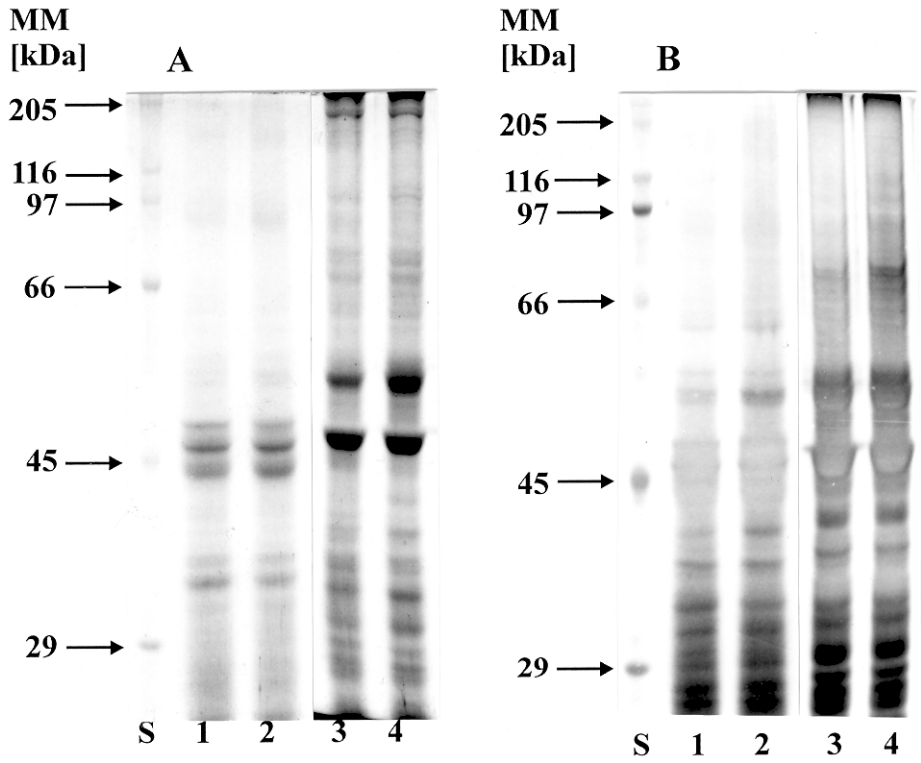


**Figure 1.** SDS-PAGE of chicken liver proteins cross-linked to DNA by *cis*-DDP (lanes 1 and 2, two separate preparations) and total nuclear proteins (lanes 3 and 4, two separate preparations) on 8% acrylamide slab gels. Gels were stained with Coomassie Brilliant Blue R-250 (A) and with silver method (B). 50 $\mu$g (A) or 25 $\mu$g (B) proteins were applied *per* gel. S, molecular mass markers.

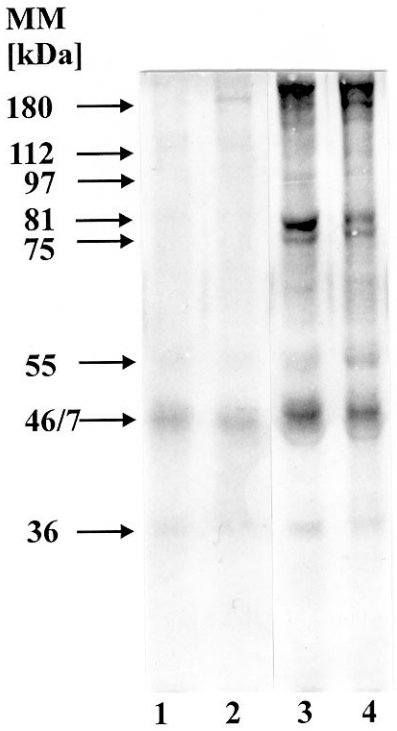


**Figure 2.** Chicken liver proteins cross-linked to DNA by *cis*-DDP (lanes 1 and 2, two separate preparations, 50 $\mu$g) and total nuclear proteins (lanes 3 and 4, two separate preparations, 50 $\mu$g) electrophoresed on 8% acrylamide slab gels were transferred onto Immobilon-P and tested for GNA binding.

Then, intact nuclei were incubated with 1 mmol/l *cis*-DDP for 2 h at 37℃ and proteins cross-linked to DNA were isolated by the technique developed by Ferraro et al. (1991). Nuclear proteins were separated by one-dimensional electrophoresis performed in slab polyacrylamide gels containing 0.1% SDS and 8% acrylamide (pH 8.8) with 3% stacking gel (pH 6.8) according to Laemmli (1970). Typical polypeptide profiles of proteins cross-linked to DNA by *cis*-DDP and their comparison with the profiles of total nuclear proteins obtained by staining with Coomassie Brilliant Blue R-250 and silver are shown in Fig. 1. The main proteins cross-linked to DNA by *cis*-DDP in chicken liver nuclei have molecular masses in the range from 29 to 66 kDa.

The proteins of whole nuclei and proteins cross-linked to DNA by *cis*-DDP separated by SDS-polyacrylamide slab gel electrophoresis were transferred onto Immobilon-P membrane and tested for lectin binding (for details see Materials and Methods). The results of these experiments are demonstrated in Figs. 2–4. It was observed that *Galanthus nivalis* agglutinin reacts with glycoproteins cross-linked to DNA with molecular masses about 36, 46/7 and 55 kDa (Fig. 2.). This kind of recognition strongly indicates a high mannose type of oligosaccharide. The presence of terminal non-reducing galactose residues in nuclear glycoproteins is shown

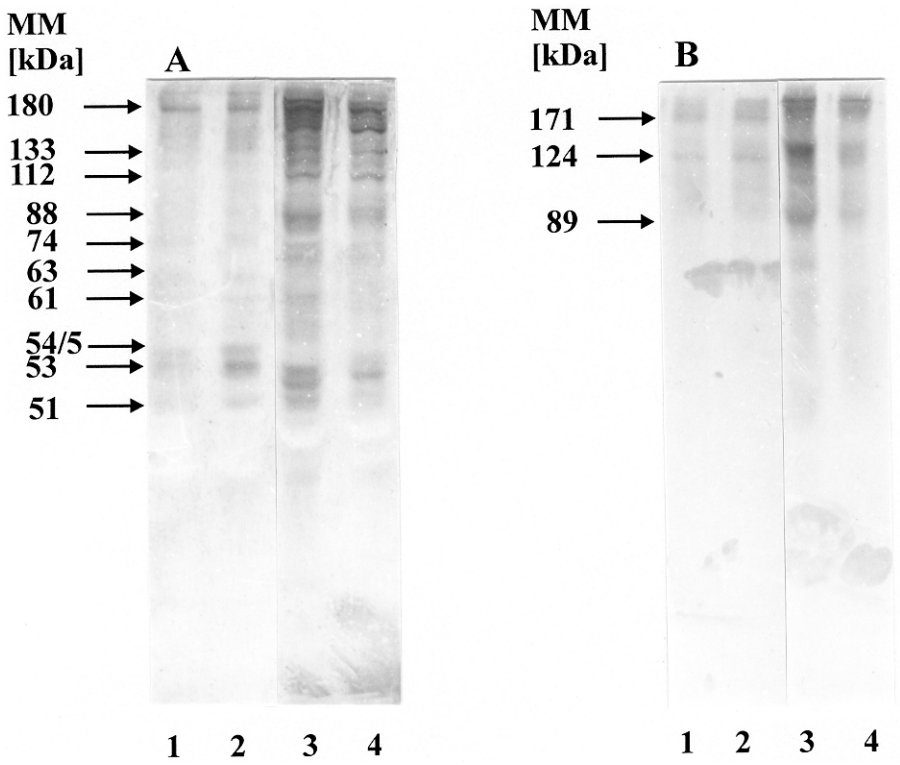


**Figure 3.** Chicken liver proteins cross-linked to DNA by *cis*-DDP (lanes 1 and 2, two separate preparations, 50 $\mu$g) and total nuclear proteins (lanes 3 and 4, two separate preparations, 50 $\mu$g) electrophoresed on 8% acrylamide slab gels were transferred onto Immobilon-P and tested for RCA-120 (A) and DSA (B) binding.

by the interactions with *Datura stramonium* and *Ricinus communis* agglutinins. RCA-120 recognized seven glycoproteins with molecular masses in the range from 51 to 180 kDa and DSA binds to glycoproteins with molecular masses 124 and 171 kDa (Fig. 3). *Sambucus nigra* as well as *Maackia amurensis* agglutinins recognize sialic acid residues bound to galactose, but SNA is specific for $\alpha$(2-6) linkage whereas MAA for $\alpha$(2-3) linkage. It is remarkable that most of chicken liver nuclear glycoproteins cross-linked to DNA were recognized by SNA and only one by MAA. These results suggest that $\alpha$(2-6) type of linkage is characteristic for glycoproteins of this fraction rather than $\alpha$(2-3).

## Discussion

For many years the dogma in glycobiology had been that nuclear and cytoplasmic proteins were not glycosylated. Now, it is known that glycoproteins are in-

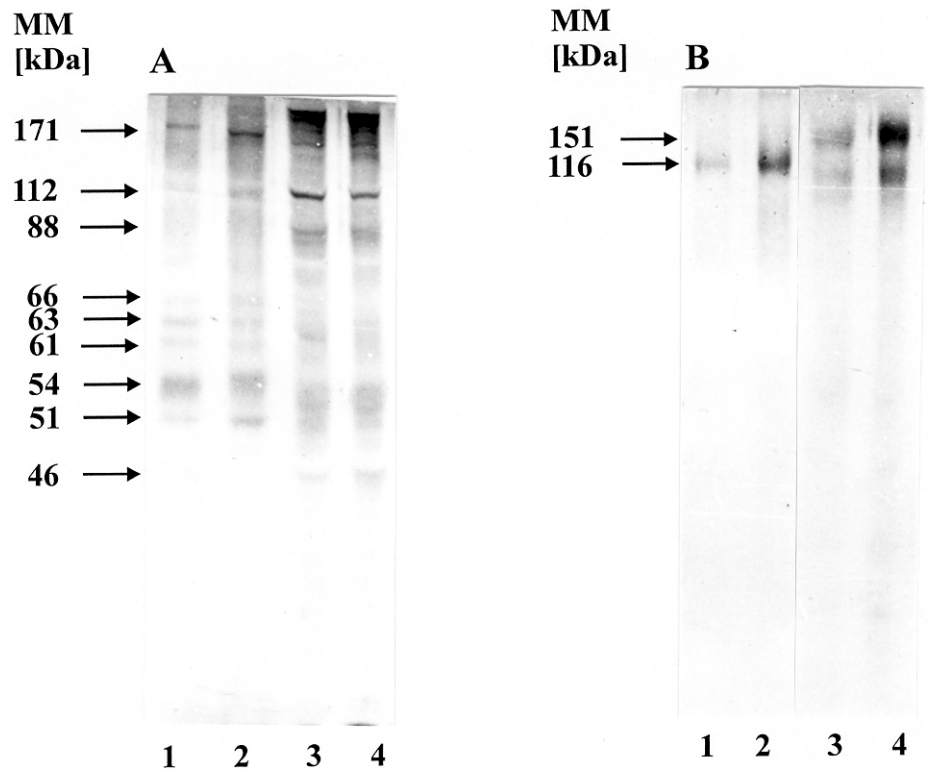


**Figure 4.** Chicken liver proteins cross-linked to DNA by *cis*-DDP (lanes 1 and 2, two separate preparations, 50 $\mu$g) and total nuclear proteins (lanes 3 and 4, two separate preparations, 50 $\mu$g) electrophoresed on 8% acrylamide slab gels were transferred onto Immobilon-P and tested for SNA (A) and MAA (B) binding.

tegral components of animal cell nuclei. In fact, the nuclear glycoproteins that have been identified unambiguously are those bearing single *N*-acetylgucosamine residues linked to the hydroxyls of serine or threonine residues on proteins by *O*-glycosidic linkage (*O*-Glc*N*Acylation). Many nuclear proteins have been identified to carry this modification including RNA polymerase II and its transcription factors, nuclear pore proteins, oncogene products and tumor supressors (for recent reviews, see Comer and Hart 1999, 2000; Wells et al. 2001). The presence of other glycoproteins bearing complex oligosaccharide chains (classical *N*-glycans) in cell nucleus is sometimes subject of criticism, and some authors suggest that they arise from extranuclear contamination of the nuclei during isolation procedure (Medina and Haltiwanger 1998). However, numerous valuable papers can be cited which provide evidence on the presence of nuclear proteins modified with complex carbohydrate structures (Codogno et al. 1992; Eufemi et al. 1992; Ferraro et al. 1994; Rousseau et al. 1997).

Our previous lectin binding studies on nuclear glycoproteins of hamster, chicken and frog liver cells showed existence of many *N*-glycosylated proteins in chromatin and nuclear matrix (Lipińska et al. 1994; Krześlak and Lipińska 1999, 2001). In the present study we used DNA-protein cross-linking reaction to obtain, among others, additional confirmation of intranuclear location of glycoproteins. *cis*-DDP was chosen as the cross-linking agent because of its propensity to cross-link DNA to nonhistone proteins rather than to histones (Cervoni et al. 1999).

The main chicken liver proteins cross-linked to DNA by *cis*-DDP separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 or silver were characterized by molecular masses in the range from 29 to 66 kDa. Our results are in agreement with the results of Ferraro et al. (1996) who showed that among the proteins isolated from cross-linked complexes in chicken liver nuclei relatively abundant were those with molecular masses 38, 40, 51, 66 and 68–70 kDa. These authors identified some of these proteins as components of the nuclear matrix.

It is known that glycoproteins stain weakly in polyacrylamide gels by commonly used protein stains such as Coomassie Brilliant Blue R-250 or silver, probably due to steric interference (Møller and Poulsen 1995). Thus, some glycoprotein bands, which were undetected by protein staining with Coomassie Brilliant Blue R-250 and silver, were revealed by lectins.

The use of lectins with different sugar specificity allowed us to draw some conclusions about sugar component of nuclear glycoproteins from chicken liver cells. Our results showed that among the proteins cross-linked to DNA there are glycoproteins bearing high mannose type of oligosaccharide as well as glycoproteins with complex type of oligosaccharide, which, besides mannose contain also *N*-acetylglucosamine, galactose and sialic acid residues.

The evidences showing that *N*-glycosylated proteins can be cross-linked to DNA in intact nuclei or cells strongly argues for existence of nuclear *N*-glycosylated proteins even if the biosynthesis of such glycoproteins has not yet been elucidated. The major question how carbohydrate moieties contribute to nuclear glycoprotein structure and function is not entirely clear, either. Now it is known that some animal endogenous lectins are located in nuclei (Wang et al. 1991; Hubert and Sève 1994; Felin et al. 1997; Roussseau et al. 2000). Some of them are also glycosylated (e.g. CBP70) (Rousseau et al. 1997). It is suggested that lectins can modulate nuclear activities such as DNA transcription, splicing and RNA transport by specific interactions with nuclear glycosylated proteins.

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