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The Two Isoforms of the 90-KDalton Nucleolus Organizer Region Autoantigen (Upstream Binding Factor) Bind with Different Avidity to DNA Modified by the Antitumor Drug Cisplatin

*Jordi Codony-Servat, Ramon Gimeno, Carmen Gelpi, Jose Luis Rodriguez-Sanchez and Candido Juarez**

DEPARTMENT OF IMMUNOLOGY, HOSPITAL DE SANT PAU, UNIVERSITAT AUTONOMA DE BARCELONA, AVDA. SANT ANTONI MARIA CLARET 167, BARCELONA 08025, SPAIN

ABSTRACT. It has been previously described that some proteins containing HMG boxes are able to bind more strongly to DNA modified with cis-diamminedichloroplatinum(II) (cisplatin) than to unmodified DNA. In the present study, we analyzed the interaction of cisplatin-modified DNA with the human autoantigen NOR-90 (UBF), a transcription factor that contains several HMG boxes. Using autoantibodies against NOR-90 to perform ELISA and immunoprecipitation, it was confirmed that NOR-90 (UBF) was able to bind cisplatin-modified DNA more avidly than unmodified DNA or trans-diamminedichloroplatinum(II) (transplatin) modified DNA. Moreover, by Southwestern, we observed that the 97 kDalton isoform of NOR-90 (UBF1) was able to bind cisplatin-modified DNA more strongly than the 94 kDalton isoform (UBF2); binding of unmodified DNA or transplatin-modified DNA was not detected with either isoform. Sera containing autoantibodies against NOR-90 did not inhibit, but increased the binding of NOR-90 to cisplatin-modified DNA. BIOCHEM PHARMACOL 51;9:1131–1136, 1996.

KEY WORDS. NOR-90; UBF; cisplatin; DNA adducts; HMG domains

Cisplatin† is an antitumor drug used for the treatment of many human cancers, including testicular, ovarian, head and neck, bladder, esophageal, and small-cell lung cancers. Cellular DNA is generally thought to be the critical biological target for cisplatin-mediated cell killing [1]. A number of different intrastrand and interstrand DNA adducts are formed after cisplatin exposure of mammalian cells, with the 1,2-intrastand d(GpG) and d(ApG) being the main adducts formed *in vitro*. The intrastrand cisplatin cross-link produces a local distortion of DNA and allows HMG1 and other proteins containing HMG boxes to increase their affinity to DNA containing a cisplatin adduct [2–8].

Several years ago, we described the presence of autoantibodies against a new protein, NOR-90, detected in the sera of patients with autoimmune diseases [9]. This protein was located by immunofluorescence in nucleolus-organizing regions (NOR), which contain many copies of the gene that codifies ribosomal RNA. NOR-90 and the human upstream binding factor (hUBF), a rRNA transcription factor containing several HMG domains, were identified when NOR-90 was cloned by Tan and coworkers [10, 11]. Recently, it has been shown that UBF (NOR-90) binds cisplatin adducts with high specificity [12].

In contrast with other members of the HMG box protein family, NOR-90 (UBF) is unique in having more than two HMG boxes, which are required for DNA binding. This protein has two isoforms, NOR-97 (UBF1) and NOR-94 (UBF2). NOR-94 (UBF2) differs from NOR-97 (UBF1) in that it lacks 37 amino acids within the second HMG box. Because the two isoforms show different binding affinity to ribosomal gene promoter and enhancer [13], we speculate that these two isoforms also show different binding affinity to cisplatin-modified DNA (cisPt-DNA) in correlation with that observed for ribosomal DNA. Our data support this hypothesis, indicating that NOR-97 (UBF1) binds cisplatin adducts more strongly than NOR-94 (UBF2).

MATERIALS AND METHODS

Sera

Sera from 5 different patients with the anti-NOR specificity, 10 normal human sera (NHS), and sera with autoantibodies against DNA (3), histones (4), HMG proteins (2), and other DNA binding proteins (5), obtained from our serum bank, were used in these studies.

* Corresponding author. Tel. 343 291 9017; FAX 343 291 9066.

† *Abbreviations*: cisplatin, cis-diamminedichloroplatinum(II); transplatin, trans-diamminedichloroplatinum(II); cisPt-DNA, cisplatin-modified DNA; transPt-DNA, transplatin-modified DNA; NOR, nucleolus organizer region; UBF, upstream binding factor; HMG, high-mobility group.

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Nuclear Extracts

Nuclear extracts were prepared basically as previously described [14]. THP1 cells (human acute monocytic leukemia cell line, American Tissue Culture Collection, Rockville, MD, U.S.A.) were washed in 10 mM sodium phosphate (pH 7.2) and 150 mM NaCl (PBS), resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)), and allowed to stand for 10 min. An equal volume of buffer A containing 0.2% Nonidet P-40 (NP-40) was then added. After swelling for 15 min, nuclei were spun down and the cytoplasms discarded. Nuclei were resuspended in buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and rotated for 2 hr. After centrifugation at 10,000 *g* for 5 min at 4°C, the supernatants were collected, labeled as nuclear extracts, and kept at −80°C. The protein content of the supernatants was determined by the Bio Rad Protein Assay (Bio Rad, Richmond, CA, U.S.A.). In some experiments, THP1 were grown overnight in a methionine-deficient medium supplemented with [^{35}S] methionine (5 μCi/mL, Amersham Corp., Buckinghamshire, England) to radiolabel the cellular proteins.

ELISA on DNA

For screening sera for the presence of autoantibodies against nuclear proteins that interacted more strongly with cisPt-DNA than with DNA or transPt-DNA, we used a modification of an ELISA that we had reported on previously [15]. Thymic cells were centrifuged in microtiter plates (30,000 cells/well), the supernatant was discarded and the wells allowed to dry. Then, 300 μL/well of methanol was added and allowed to evaporate until the wells were dry. 300 μL of 0.1 M HCl was added to each well and, after 60 min, the supernatant was discarded and the wells washed 3 times with PBS. Next, 100 μL of 1 mM Tris/HCl (pH 7.5), 3 mM NaCl, or the same buffer containing 0.03 μg/mL of cisplatin (Aldrich, Chem. Co., Milwaukee, WI, U.S.A.) or 0.03 μg/mL of transplatin (Aldrich, Chem. Co.) were added to the corresponding wells in duplicate and allowed to stand overnight at 37°C, protected from light. The wells were washed with 3 changes of 1 mM Tris/HCl (pH 7.5), 3 mM NaCl, 10 min each, and incubated with blocking solution (10 mM Tris (pH 7.5), 0.4 M NaCl) containing 5 mg BSA/mL. Duplicates were incubated with blocking solution containing 1 mg BSA/mL (buffer A) or with the nuclear extract overnight at 4°C. The wells were washed with buffer A and incubated with the sera to be tested, diluted 1/200 in 10 mM Tris/HCl (pH 7.5), 0.4 M NaCl, 0.1% Tween 20 and 1% fetal calf serum for 2 hr at room temperature. The wells were washed again with buffer A and incubated with a 1/500 dilution of peroxidase-labeled rabbit immunoglobulins to human immunoglobulins (Dako, Glostrup, Denmark) for 3 hr. Finally, the ELISA was developed as described [15].

Western Assays

Nuclear extracts were fractionated on SDS-polyacrylamide gels as described [16] and transferred electrophoretically to nitrocellulose paper [17] (Bio-Rad). Transferred proteins were allowed to renature in binding buffer: 20 mM Tris/HCl (pH 7.5), 60 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, DTT 1 mM, and 8% glycerol. The paper was then blocked with 5% nonfat dry milk in 10 mM Tris/HCl (pH 7.5), 0.15 M NaCl (TBS), and incubated with patients' sera or NHS diluted 1/200 in TBS containing 3% nonfat dry milk. Rabbit antihuman immunoglobulins (1/200, Dakopatts), peroxidase-labeled swine antirabbit immunoglobulins (1/500, Dakopatts) and the Bio-Rad developing reactive (Bio-Rad) were used to detect the antigen.

Labeling of DNA

DNA from rat liver was obtained in our laboratory by hydroxyapatite chromatography and sonicated to obtain a media size of approximately 260 basepairs. DNA was labeled with [α-^{32}P]dCTP by Nick translation (Nick translation kit, Amersham International plc, Buckinghamshire, England). Labeled DNA was modified with cisplatin or transplatin (1 μg/mL) in 1 mM sodium phosphate (pH 7.0), 10 mM NaCl, for 15 hr at 37°C, protected from light.

Southwestern

Nuclear extracts were fractionated in polyacrylamide gels, transferred to nitrocellulose paper, and allowed to renature in binding buffer as described in the Western section. Blots were washed in binding buffer containing 0.25% nonfat dry milk (buffer B) and incubated for 15 min in the same buffer containing 4 μg/mL of sonicated calf thymus DNA (Pharmacia, Uppsala, Sweden). Labeled DNA was then added and incubated for 2 hr at room temperature. Blots were washed 3 times in binding buffer containing 0.4 M NaCl (0.2 M NaCl was used for the analysis of the purified NOR-90) and, after a further wash in the same buffer without milk, blots were prepared for autoradiography.

In some experiments, blots containing transferred proteins and blocked with nonfat dry milk were incubated with sera from patients with anti-NOR-90 autoantibodies or NHS (1/50 dilution in buffer B) for 3 hr at room temperature, washed 3 times in buffer B, and incubated with the labeled DNA as before.

DNA Immunoprecipitation

DNA was labeled with [α-^{32}P]dCTP by Nick translation, as above, and distributed in 3 fractions: one was used as DNA control, another was modified with cisplatin, and the third with transplatin, as described above. These DNAs were incubated with nuclear extract for 1 hr and then immunoprecipitated with NHS or anti-NOR-90 sera, as previously described [18]. Protein-A Sepharose beads were, finally,

washed 3 times in buffer C and the radioactivity incorporated in the pellets was counted.

Purification of NOR-90

Protein-A Sepharose beads (Pharmacia) were incubated with sera containing anti-NOR-90 autoantibodies overnight at 4°C. After washing in TBS, immunoglobulins were covalently bound to Protein-A beads by incubation with glutaraldehyde (0.5% final concentration) for 30 min at room temperature. Beads were washed 3 times in 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.05% NP-40 and incubated in the same buffer containing 5 mg BSA/mL for 30 min at room temperature. Afterwards, the beads were incubated with nuclear extract and preabsorbed with NHS coupled to Protein-A Sepharose for 2 hr at 4°C. After washing in buffer C, NOR-90 antigen was eluted from the beads in 80 mM Tris/HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol for 5 min in a boiling bath and run in a SDS-polyacrylamide gel.

RESULTS AND DISCUSSION

The NOR-90 Antigen Binds cisPt-DNA with High Affinity

Figure 1 shows that the two isoforms of 94 and 97 kDaltons described for the NOR-90 antigen (UBF) are present in the nuclear extract used in our studies. It can be seen that all the sera used here (a fifth anti-NOR serum used is not shown in the figure but gave the same pattern) showed similar reactivity with both isoforms, indicating that similar amounts of both species were present in our nuclear extracts. The other common lower molecular species detected by these antisera were probably degradation products, as has been previously described [11]. When we analyzed these sera containing the anti-NOR-90 specificity and other sera with different antinuclear specificities, by means of the ELISA on DNA (see Materials and Methods), the anti-NOR autoantibodies showed a clear increase in their binding to the cisPt-DNA (Fig. 2) as compared with unmodified DNA or transPt-DNA. This increase was dependent on the presence of the nuclear extract because it was not observed in the wells in which nuclear extract was not added (data not shown). This increase was also observed with sera containing anti-HMG autoantibodies, as expected, but not with sera containing other specificities. Anti-DNA antibodies gave similar reactivity with cisPt-DNA, transPt-DNA or DNA (data not shown).

Moreover, these data obtained by ELISA were confirmed by immunoprecipitation of labeled DNAs. The anti-NOR-90 antisera immunoprecipitated a higher amount of cisPt-DNA than DNA or transPt-DNA (Fig. 3). When immunoprecipitation was performed with different NHS, only background counts (less than 300 counts) were obtained, whether labeled DNA, cisPt-DNA, or transPt-DNA were used (data not shown).

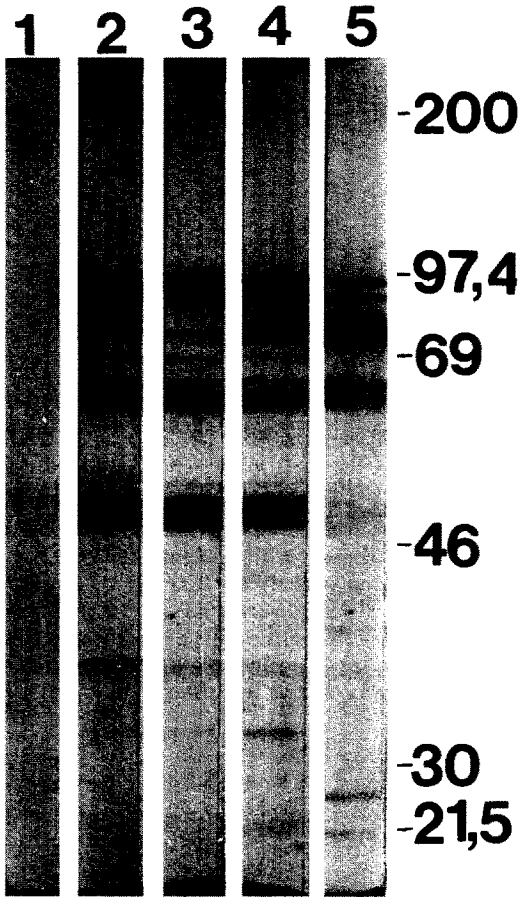

FIG. 1. Western blot of the anti-NOR-90 sera used. Nuclear extract from THP1 cells was fractionated in 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Strips were incubated with NHS (lane 1) or sera from 4 different patients containing anti-NOR-90 autoantibodies (lanes 2–5). The molecular mass of standards (kDaltons) are shown on the right.

Reactivity of THP1 Nuclear Proteins with cisPt-DNA

Because we used anti-NOR-90 sera from several patients, and all showed higher binding to the cisPt-DNA than to DNA control or transPt-DNA, we concluded that the affinity of NOR-90 for DNA increased when DNA was modified with cisplatin. Nevertheless, we could not be certain that the extracted NOR-90 antigen was not associated with other proteins, one of which could be responsible for the increase in affinity observed.

To analyze this possibility, and to determine if both NOR-90 isoforms were able to increase their affinity for DNA, we performed Southwestern blotting. Figure 4, lane 1, shows that cisPt-DNA was able to bind, as previously described, a protein of 105 kDaltons (4), as well as the 28–26.5 kDalton HMG proteins (5–6). Nevertheless, we also observed reactivity with a protein of 97 kDaltons and other lower species. Because the same blot, cut in strips, was

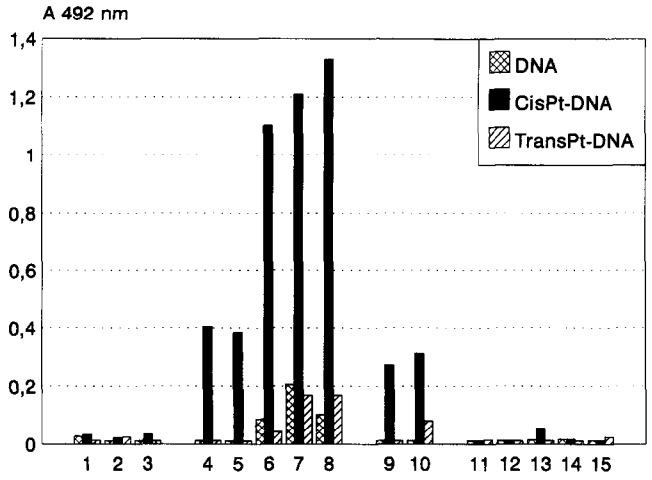

FIG. 2. ELISA detection of NOR-90 antigen. DNA in wells were left untreated, treated with cisplatin, or with transplatin and allowed to interact with nuclear extract. Afterwards, wells were incubated with NHS (bars 1–3), anti-NOR-90 sera (bars 4–8), anti-HMG sera (bars 9, 10), or sera from patients containing other antinuclear specificities (bars 11–15). Results are expressed as mean absorbance units of triplicates. The SEM was always less than 10%.

used for both Southwestern and Western, we were able to put the strips side by side and confirm that the electrophoretic mobility of the species recognized by anti-NOR-90 autoantibodies and the electrophoretic mobility of the species able to bind cisPt-DNA were identical. Neither of these species, neither the 105 kDaltons nor the HMG proteins, were detected by Southwestern when the blot was incubated with DNA or transPt-DNA (data not shown), confirming that DNA modified with cisplatin has a higher affinity for all these proteins.

The data obtained from Southwestern and Western blots

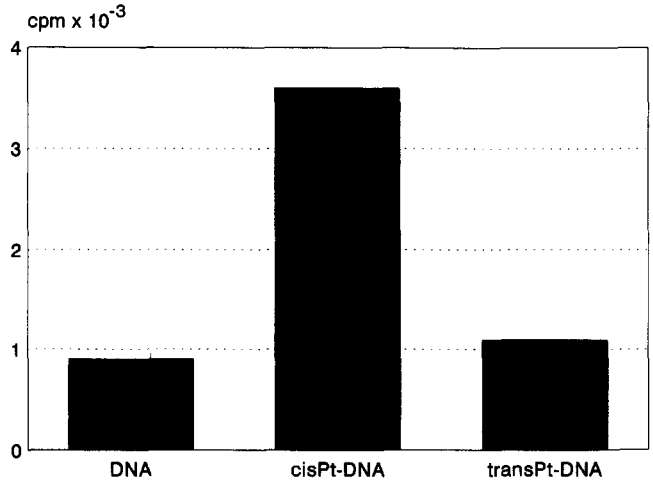

FIG. 3. Immunoprecipitation of NOR-90 antigen. DNA, cisPt-DNA, or transPt-DNA were labeled by Nick translation and immunoprecipitated by anti-NOR-90 autoantibodies as indicated in Material and Methods. Results are expressed as mean cpm of triplicates. The SEM was always less than 10%.

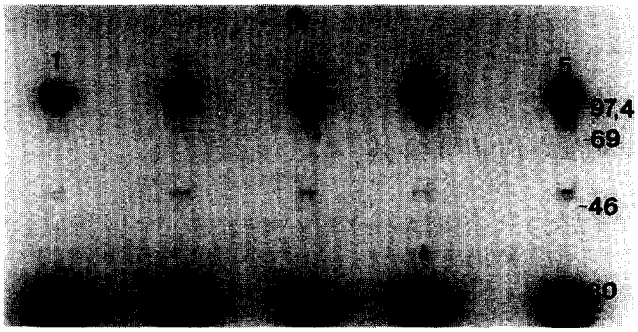

FIG. 4. Reactivity of THP1 nuclear proteins with cisPt-DNA. THP1 nuclear extracts were fractionated in a 7.5% SDS-polyacrylamide gel and transferred to nylon. After blocking, strips were allowed to stand (lane 1) or incubated with 2 different normal human sera (lanes 2, 3) or 2 different anti-NOR-90 sera (lanes 4, 5). ^{32}P-labeled cisPt-DNA was added to all strips. The molecular mass of standards (kDaltons) is shown on the right.

supported the hypothesis that the isoform of 97 kDaltons, as well as the degradation products of the NOR-90 antigen, increased their affinity for DNA when the latter was modified by cisplatin. We tried to inhibit the binding of the labeled cisPt-DNA to these bands in Southwestern to confirm our conclusion. Nevertheless, the incubation of the blots with anti-NOR-90 sera did not inhibit the binding of the cisPt-DNA to any of the species, but increased the intensity of the 97 kDalton band and allowed the appearance of a new 94 kDalton band able to bind cisPt-DNA (Fig. 4, lanes 4 and 5). When compared with the Western, this band corresponded to the low isoform of NOR-90. This effect was not observed with NHS (Fig. 4, lanes 2 and 3). We are as yet unable to explain the increase observed in the reactivity of the NOR-90 isoforms after their binding by antibodies, but we hypothesize that autoantibodies could induce some conformational changes for the effect observed. We are presently continuing our studies to confirm this hypothesis.

NOR-97 Binds cisPt-DNA More Strongly than NOR-94

Because of the results obtained in the inhibition studies, we decided to purify NOR-90 before the Southwestern assays. NOR-90 was purified from a nuclear extract by immunoprecipitation with anti-NOR-90 antibodies, run in a 7.5% SDS-polyacrylamide gel, and electrophoretically transferred to nitrocellulose. The blot was then incubated with DNA, cisPt-DNA, or transPt-DNA previously labeled by Nick translation with [α-^{32}P]dCTP. As shown in Fig. 5A, lane 2, cisPt-DNA was able to bind two proteins of 97 and 94 kDaltons, the mobility of which coincided exactly with the doublet recognized by the anti-NOR-90 autoantibodies. The reactivity of the 97 kDalton band was much stronger than the 94 kDalton band. None of these species was recognized when the strips were incubated with DNA or transPt-DNA (Fig. 5A, lanes 1 and 3). Both isoforms were

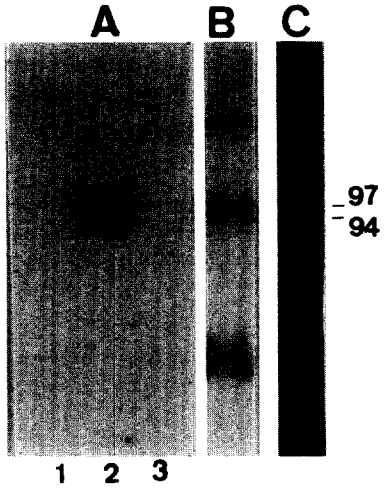

FIG. 5. Reactivity of purified NOR-90 isoforms with cisPt-DNA. NOR-90 was purified from a THP1 nuclear extract, [^{35}S]methionine-labeled by immunoabsorption, run in a 7.5% SDS-polyacrylamide gel, and transferred to nitrocellulose. (A): Strips were incubated with [^{32}P]-labeled DNA (lane 1), cisPt-DNA (lane 2), or transPt-DNA (lane 3). (B): The strip was incubated with anti-NOR-90 and the antigen detected as in Fig. 1. (C): The strip was directly autoradiographed to show the amount of labeled NOR-90 isoforms. The molecular mass of the bands (kDaltons) is shown on the right.

present at similar concentrations, as shown first by the similar reactivity of both isofroms in Western blot using anti-NOR-90 sera (Fig. 5B) and second by the similar amount of radioactivity detected for each isoform when, as is the case shown in Fig. 5, the nuclear extract was labeled with [^{35}S]methionine (Fig. 5C). All these data confirm that NOR-97 (UBF1) binds cisplatin-DNA adducts more strongly than NOR-94 (UBF2).

The difference in the binding affinities between NOR-97 and NOR-94 for cisPt-DNA correlated with the different binding of the two isoforms to ribosomal gene promoter and enhancer, and with their transcriptional activity. In effect, it was shown that UBF1 has a high affinity for ribosomal DNA (rDNA) [13] and is a potent transcriptional activator and antirepressor, whereas the ability of UBF2 to bind rDNA and its ability to mediate transcriptional activation and antirepression, both *in vivo* and *in vitro*, was at least one order of magnitude lower. Therefore, the ability of cisPt-DNA to "mimic" the structural features of rDNA that determine the different affinity of the two isoforms of NOR-90 justifies the importance of the alterations observed. Moreover, because of its higher affinity for NOR-97/UBF1, cisplatin-DNA adducts were able to produce a marked decrease in the amount of this isoform available for rDNA binding without affecting NOR-94/UBF2, the transcription inactive isoform.

In conclusion, results from the present study indicate that NOR-97/UBF1 is able to bind cisPt-DNA with a higher affinity than NOR-94 (UBF2). This could have significant implications in the response of the cells to cisplatin. Indeed, the localization of UBF in some "wrong places" could deregulate the transcription of several genes. Furthermore, the strong binding of UBF1 for cisplatin adducts could reduce the amount of this isoform available for rDNA binding, disrupting the regulation of rRNA synthesis. Because of the major role of UBF/NOR-90 in the transcription machinery of RNA polymerase I, it could be of great relevance to know if there is an anomalous expression of transcripts by RNA polymerase I as a consequence of the treatment of cells with cisplatin.

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