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A Simple Protocol for the Expression and Purification of NC2

[1]Diana Karina Guillén, [1]Nadine Möbius,
[2]Luís Carlos Rodríguez and [1]Enrique Castaño
[1]Unidad de Bioquímica y Biología Molecular de Plantas,
Centro de Investigación Científica de Yucatán, Calle 43 No. 130,
Col. Chuburna de Hidalgo, CP 97200, Mérida, Yucatán, México
[2]Unidad de Biotecnología, Centro de Investigación Científica de Yucatán,
Calle 43 No .130, Col. Chuburna de Hidalgo,
CP 97200, Mérida, Yucatán, México

Abstract: Transcriptional regulation depends on the appropriate set of positive and negative regulating signals in order to provide the correct gene expression. Studies in eukaryotic gene expression over the last few years have shown that NC2 acts as a general repressor of transcription. Functional *in vitro* studies require large amounts of highly purified recombinant protein. The case of rNC2a (Drap1) which up to date has been difficult to express in bacteria is common among many eukaryotic proteins. Moreover, under current protocols both subunits have to be denature in urea after combination and allow a renaturation process which may render many inactive NC2 complexes. Here we provide a simple protocol that includes a new expression vector and the protocol for over expressing and purifying both subunits in a native state.

Key words: Dr1, drap1, NC2, repressor, transcription, gene regulation

INTRODUCTION

Gene activation and repression has been under intense study during the last three decades. In the early 90's, a repressing activity was found in HeLa nuclear extract that could selectively reduce basal transcription but did not affect the activated transcription. Consequently, this activity was named Negative Cofactor 2 (NC2) (Inostroza *et al*., 1992). Soon after, protein Dr1 was found to be a general transcription repressor, particularly when it is associated to its partner Drap1. Once NC2α/β clones where sequence they were found to be the same as Dr1/Drap1 (Goppelt *et al*., 1996). NC2 is composed of two subunits NC2α 22 kDa and NC2β 20 kDa. The amino acid sequence of NC2α and β are related to the Histones H2A and H2B, forming the histone motif which allow them to heterodimerize (Tresaugues *et al*., 2006; Goppelt *et al*., 1996). This motif is common to a series of factors involved in gene regulation and chromatin organization.

The recombination NC2 proteins have been found to repress transcription in vitro by interacting with TBP thereby preventing TFIIB from forming a DNA-TBP-TFIIB complex. The result is the inhibition of the preinitiation complex formation while maintaining a DNA-TBP-NC2 complex (Goppelt *et al*., 1996; Kim *et al*., 1995; Gilfillan *et al*., 2005). TFIIA competes with NC2 for TBP and allows TFIIB to bind, which promotes binding from the rest of general transcription factors and RNA Pol II to the promoter and for transcription initiation to occur (Roeder, 2005; Castaño *et al*., 2006). Furthermore, the crystal structure has been carried on a portion of the NC2 complex interacting with

Corresponding Author: Enrique Castaño, Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburna de Hidalgo, CP 97200, Mérida, Yucatán, México

TBP (Kamada *et al*., 2001). Available data has shown such interacting domains of NC2 with TBP, consistent with the biochemical binding experiments and its activity as a repressor. As NC2 has been shown over the years to be of a general transcriptional repressor of class II genes; however, in order to achieve a thorough understanding of the function of this repressor, *in vitro* experiments require a reliable source of material. Solutions to this problem included the removal of portions of the protein as in the case of the crystallographic study (Kamada *et al*., 2001) 21 unrelated amino acids where added to increase the protein yield.

Since the current systems for expressing NC2 subunits produce a low yield NC2α as seen by others and in our present experiments that in the absence of a partner, (Naar *et al*., 2001; Bikle *et al*., 2003).

Here, we present a simple non-denaturing protocol for obtaining high yield active rNC2 as well as a simple protocol for its purification. This may be applicable for the expression of other complex protein systems.

MATERIALS AND METHODS

Vectors

The vector pNC2β-6HIS and pNC2α-6HIS have previously been described (Bikle *et al*., 2003). The vector pNC2α-M2 was carried out by subcloning NC2α with Nde I and BamH I into pET11d-flag. The final co-expressing vector was carried out by subcloning NC2α-M2 with Bgl II and Hind III in to pNC2β-6-HIS, in order to generate pNC2α-M2/NC2β-6HIS. pECHIV-111/80 has been previously described (Bikle *et al*., 2003).

Protein Expression and Purification

Protein Expression

Sequences of NC2α and NC2β are in vector pET11d for expression both vectors were transformed in *E. coli* BL21(DE3) pLysE. from Invitrogen and then allowed to grow to an OD of 0.5 at 600 nm. Next, IPTG (Isopropylthiogalactoside) 1 mM was added and incubated at 30°C for 3 h. (30°C reduces the level of protein degradation and increases the overall yield). Followed by 10 min of centrifugation at 4000 x g. In the case of NC2α after collecting the bacterial pellet 5 mL of denaturing buffer (20 mM Tris HCl, pH 7.9, 8M Urea, 0.1M NaH_2PO_4, 0.5 M KCl, 20 mM Imidazol) was added and cells were sonicated 3 times. The re-suspended lysate was centrifuged at 4000 x g for 10 min to remove cell debris and the supernatant allowed binding to 0.1 mL of Ni^{2+}-nitrilotric acetic acid resin for one hour, the column was then allowed to flow by gravity and wash with 5 mL of the denaturing buffer. Finally 0.3 mL of elution where recover in a denaturing buffer containing 250 mM Imidazole.

For the expression of NC2β and the co-expression M2:NC2α-6His: NC2β system the bacterial pellets were resuspended in 5 mL of Lysis buffer 20 mM Tris HCl, pH 7.9, 1 μg mL^{-1} Lyzosyme, 0.5 M KCl, 20 mM Imidazole, PMSF (Phenylmethyl sulfonyl fluoride) 4 mM, 20% Glycerol) sonicated 3 times on ice. The lysate was centrifugated 4000 x g for 10 min and the supernatant allowed binding in 0.1 mL of Ni^{2+}- nitrilotric acetic acid resin for 1h in ice, the column was then allowed to flow by gravity and wash with 5 mL lysis buffer. The elution was carried out by adding 0.3 mL of elution buffer (20 mM Tris HCl, pH 7.9, 0.4 M KCl, PMSF 4 mM, 250 mM Imidazole).

Second step purification utilizing 0.1 mL M2-resin was used to further purify the NC2 complex in BC100 buffer, 1 h incubation at 4°C of the resin with the Ni^{2+} purified eluate was followed by 3 wash steps of BC100 and finally eluted after incubation with 0.2 mL of BC100 (20 mM Tris HCl, pH 7.9 at 4°C, 20% glycerol, 0.2 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol, 0.5 mM PMSF and 0.1 M KCl) with 0.2 mg mL^{-1} flag peptide (DYKDDDDK) from Sigma for 30 min at 4°C.

Transcription *in vitro*

Methodology published in Castaño *et al.* (2000). Brief explained a reaction mixture containing either nuclear extracts or purified transcription factors were mix with 100 ng od pHIV-33/+68 in the presence of 0.5 mM NTP, 5 mM MgCl, 5 mM DTT, in 20 mM HEPES KOH pH 8.4, in 20 μL final volume. The transcription reactions were incubated at 30°C for one hour. A control label DNA was added to the mixture. Products were stop with the stop-buffer (20 mM Tris pH 8.0, 0.5% SDS, 150 mM NaCl, 100 ng Glycogen) and extracted in phenol, ethanol-precipitated and used for primer extension reactions utilizing ^{32}P label primer 5´GCCAAGCTATTTAGGTGACACTAT3´ in a primer extension buffer (20 u RNAasin, 1.5 mM Actinomicin D, 15 mM DTT, 12 mM $MgCl_2$, 1 mM dNTP's and 50 mM Tris pH 8.0) for 1 h at 37°C.

RESULTS

NC2 is considered to be a general negative regulator of transcription initiation; it has a unique mechanism of repression of basal transcription through direct binding of TBP therefore blocking the pre-initiation complex formation. However, several more studies have to be carried out to validate the overall complexity of transcription repression and to verify the current model of action. For this recombinant proteins are useful, however it is well known that NC2α yield production is low, our results from Fig. 1A show the same result as obtained by other authors (Kamada *et al*., 2001). Even after four hours we observed a low level expression as compared with NC2β Fig. 1. NC2β induction resulted in an increase level of expression over time, with a peak of expression between 2-4 h after the addition of 1 mM IPTG, Fig. 1B. Both polypeptides were purified with Ni-agarose resin as seen in Fig. 1C. These typical results are not new in the field and present a second problem which is to obtain a fully fictional complex NC2α/β (Gilfillan *et al*., 2005; Kamada *et al*., 2001; Yeung *et al*., 1997). The typical solution for this problem is a denaturation-renaturation procedure which may render unknown percentage of active molecules. Other solutions to the problem have been the removal of several amino acids from the C terminus of NC2α to increase stability and increase the yield. This however brings an incomplete protein which may differ in action from the complete molecule. Therefore the strategy was undertaken to express both subunits at the same time and for each to have a different tag as seen in Fig. 2A, 6His to NC2β and Flag to NC2α.

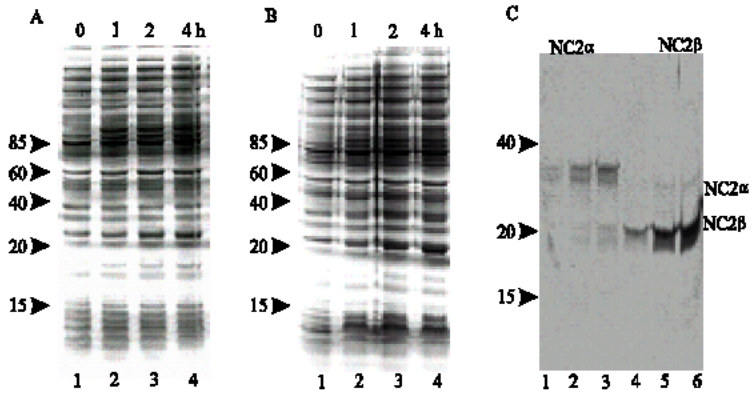

Fig. 1. A): Twelve percent Polyacrilamide gel, induction of NC2α with 1 mM of IPTG at 1, 2 and 4 h, B) Induction of NC2β with 1 mM of IPTG at 1, 2 and 4 h and C) Fifteen percent Polyacrilamide gel purification de NC2α (lanes 1-3) and NC2β (lanes 4-6) with Ni^{2+} resin eluted with 250 mM of Imidazole

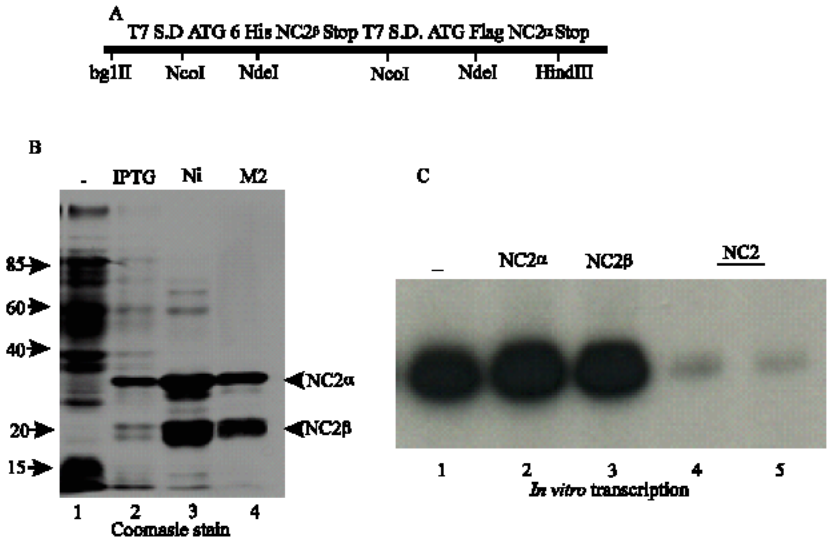

Fig. 2: A) Map of final plasmid construction containing two sequences de both proteins 6His-NC2β and Flag-NC2α. B) Poliacrylamide gel 12%, cells induced with IPTG 1 mM and wash with imidazole 250 mM by Ni^{2+} resin. Purification of proteins NC2α and NC2β with Ni agarose (lane 3) and followed of M2 resin (lane 4) and C) *in vitro* Transcription assay of transcription 100 ng of NC2β or NC2α were used in lanes 2 and 3, respectively, 30 ng of NC2 complex repressed transcription lanes 4-5

We decided to co-expressed both subunits in the same vector as seen in Fig. 2B and found that when both subunits are expressed the overall yield of NC2α recombinant protein is significant greater than when expressed individually, also the addition of a different tag allowed a second step of purification rendering a complete NC2 complex molecule without the need of denaturation steps that were used in other protocols.

Finally, we prove the functionally of the protein on an *in vitro* transcription system; Fig. 2C shows that 20 ng of rNC2 are sufficient to represses transcription as seen in lanes 4 and 5 while 100 ng of either NC2α or NC2β have no significant effect on transcription repression in this system. It should be noted that if NC2 is purified solely by Ni^{2+} column the protein co-purifies with a bacterial RNA polymerase which leads to a high level of unrelated transcripts. This is due to a co-purification of a bacterial polymerase which can lead to wrong conclusions. This can be corrected by purifying the NC2 protein over M2 resin as done in these experiments.

DISCUSSION

Transcription by RNA polymerase II in the eukaryotic cell is regulated by the complex interplay of positive and negative regulators. One of the most important general repressors of transcription NC2 was found to consist of two subunits named NC2α (Drap1) y NC2β (Dr1). NC2 binds to TBP and blocks the binding of both TFIIA y TFIIB, which depending on the overall concentrations of TFIIA it may cause a release of NC2 and an activation of transcription. Furthermore, NC2 has been shown to enhanced the ability of TBP to bind DNA, thereby showing a path for a positive role in transcription for this molecule. Over the years this simple mechanism of repression has been challenged from data of diverse laboratories which show that NC2 interacts with other molecules and may

function to block only activated transcription and repress additional transcription initiation sites (Castaño *et al*., 2000; Castaño *et al*., 2006; Colli *et al*., 2006; Klejman *et al*., 2004; Willy *et al*., 2000).

In order to solve for a consistent source of rNC2 material to further test current hypothesis we cloned both subunits of NC2 (α and β), in to pET 11d vector and found that the expression as well as subsequent purification of the complex was easier and more reliable as compared to individual purifications, moreover the expression of both subunits avoid the renaturation steps that were current norm in previous protocols and which may render an unknown amount of active protein. This simple technique of coexpresion may allow expression of others proteins like, NF-YB/ NF-YC and YBL1/YCL1 (Bellorini *et al*., 1997; Bolognese *et al*., 2000), that utilize a similar denaturation renaturation protocol and that an unknown percentage of active protein as well as an unknown level of denature proteins that may still play some effect on the tested activity, which may mislead the conclusions of the assay tested.

In the summary, we believe that this simple double expression system would be useful for other hetero-dimmer proteins where one or both of the polypeptides precipitate during bacterial expression.

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