A Simple Protocol for the Expression and Purification of NC2

1Diana Karina Guillén, 1Nadine Mobius,
2Luis Carlos Rodríguez and 1Enrique Castaño
1Unidad de Bioquímica y Biología Molecular de Plantas,
Centro de Investigacion Científica de Yucatán, Calle 43 No. 130,
Col. Chuburna de Hidalgo, CP 97200, Mérida, Yucatán, México
2Unidad 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 NC2α (Drp1) 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 area 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) (Instroza 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 NC2αβ clones
where sequence they were found to be the same as Dr1/Drap1 (Goppel 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; Goppel 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 TFII B from forming a DNA-TBP-TFII B complex. The result is the
inhibition of the preinitiation complex formation while maintaining a DNA-TBP-NC2 complex
(Goppel et al., 1996; Kim et al., 1995; Gilfillan et al., 2005). TFII A competes with NC2 for TBP and
allows TFII B 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

263
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, (Naat 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 (Isopropyl thiogalactoside) 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 NaH₂PO₄, 0.5 M KCl, 20 mM Imidazole) 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 Ni²⁺-nitrilotriacetic 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 NCβ and the co-expression M2/NC2α-6HIS: NCβ system the bacterial pellets were resuspended in 5 mL of Lysis buffer 20 mM Tris HCl, pH 7.9, 1 μg mL⁻¹ Lysozyme, 0.5 M KCl, 20 mM Imidazole, PMSF (Phenylmethyl sulfonfonyl fluoride) 4 mM, 20% Glycerol) sonicated 3 times on ice. The lysate was centrifuged 4000 x g for 10 min and the supernatant allowed binding in 0.1 mL of Ni²⁺-nitrilotriacetic 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 Ni²⁺ 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). Briefly explained a reaction mixture containing either nuclear extracts or purified transcription factors were mixed with 100 ng of HIV-334-68 in the presence of 0.5 mM NTP, 5 mM MgCl₂, 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 [32P] label primer 5’ GCGAAGCTTATTGAGCTGCGATATAACGC 3’ in a primer extension buffer (20 u RNAsin, 1.5 mM Actinomycin D, 15 mM DTT, 12 mM MgCl₂, 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 recombination 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. 1C. 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 functional complex NC2αβ (Gillilan et al., 2005; Kamada et al., 2001; Yung 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.

![Image](image-url)

**Fig. 1. A**: Twelve percent Polyacrylamide 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 Polyacrylamide gel purification de NC2α (lanes 1-3) and NC2β (lanes 4-6) with Ni²⁺ resin eluted with 250 mM of imidazole. 265
Fig. 2: A) Map of final plasmid construction containing two sequences of both proteins 6His-NC2β and Flag-NC2α. B) Polyacrylamide gel 12%, cells induced with IPTG 1 mM and wash with imidazole 250 mM by Ni²⁺ 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 functionality of the protein on an in vitro transcription system; Fig. 2C shows that 20 ng of rNC2 are sufficient to repress 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 Ni²⁺ 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 (Dnap1) and NC2β (Dr1). NC2 binds to TBP and blocks the binding of both TFIIA and TFIIIB, 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 enhance 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 PET11d 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 coexpression 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-dimer proteins where one or both of the polypeptides precipitate during bacterial expression.

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