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A Heat Shock Transcription Factor Like Protein in the Nuclear Matrix Compartment of the Tissue Cultured Mammalian Lens Epithelial Cell

M. Bagchi,* M. Katar, and H. Maisel

Department of Anatomy/Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Abstract This investigation characterizes a prominent nuclear matrix protein isolated from tissue cultured mouse lens epithelial cells. The nuclear matrix protein was isolated using a modified Penman technique. Total nuclear matrix proteins were further separated by SDS-polyacrylamide gel electrophoresis. The SDS-PAGE profile of the nuclear matrix proteins displayed a prominent doublet band at 60 kDa region. Nonequilibrium 2D gel electrophoresis revealed that this protein is a basic nuclear protein. This 60 kDa protein was further characterized by comparing its internal peptide amino acid sequence with known protein sequence using the BLAST technique, and this study demonstrated that 60 kDa nuclear matrix protein displays significant sequence similarity with Xenopus Laevis heat shock transcription factor. We also raised antibodies against 60 kDa nuclear matrix protein. Immunofluorescence, studies showed that this 60 kDa nuclear matrix protein preferably decorates nucleus, and puncted pattern of fluorescence suggest presence of this protein in the discrete areas of the nucleus. Heat shock transcription factors upregulate synthesis of heat shock proteins and many of these protein act as molecular chaperones. Thus, presence of a nuclear matrix protein with significant sequence similarity with heat shock transcription factor suggests sustained heat shock protein synthesis in the mouse lens cells. J. Cell. Biochem. 80:382–387, 2001. © 2001 Wiley-Liss, Inc.

Key words: nuclear matrix protein; heat shock transcription factor (HSF-1); molecular chaperone; lens epithelial cells; lens crystallins

The pioneering studies of Nickerson et al. [1995], and Berezney and Coffey [1976], demonstrated that the nuclear matrix (NM) is a morphologically complex network of anastomosing ribonucleoprotein strands, that participates in the three-dimensional organization of the nucleus [Fey et al., 1986]. Further investigations showed that processes involved in this nuclear packaging are of paramount importance for understanding the basic mechanisms of the gene expression [Lamond and Earnshaw, 1998]. Stein et al. [1996] demonstrated that the NM participates in gene localization by imposition of physical constraints on chromatin structure that supports the loop domains, concentration, and targeting of transcription factors. Thus, the NM plays a regulatory role in the process involved in gene expression. Piatigorsky and Zelenka [1992] clearly established that passage of an unimpeded incident light beam through the lens is dependent on optimal lens crystallin synthesis. Although many regulatory mechanisms of lens crystallin synthesis are known [Sax et al., 1997; Piatigorsky and Zelenka, 1992], little information is available about the in vivo control of possible interactions of the promotor regions of crystallin genes and discrete NM protein(s). We initiated studies to identify the major NM proteins of mammalian lens epithelial cells, and to isolate those proteins which might compartmentalize the α-crystallin gene in a specific territory of the nucleus, thus regulating its optimal expression. The results obtained from these experiments revealed that a 60 kDa doublet protein is the major constituent of the lens NM. This complex was found to have extensive amino acid sequence similarities

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*Correspondence to: Mihir Bagchi, Department of Anatomy/Cell Biology, Wayne State University School of Medicine, Detroit, MI 48201.

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with xenopus heat-shock transcription factor (XHSF-1) [Landsberger and Wolfe, 1995].

MATERIALS AND METHODS

Isolation of Nuclear Matrix Proteins

The NM proteins were isolated by the modified Penman technique [Bidwell et al., 1993] using confluent transgenic mouse lens epithelial cells (αTN4) [Yamada et al., 1990] grown in tissue culture. The proteins were extracted in a series of buffers containing detergents and nucleases to isolate the NM and the bound intermediate filament (IF) scaffold. During extraction procedure an aliquot of each fraction was collected and dissolved in SDS buffer, starting from total cellular extract of αTN4 cells to the final soluble nuclear matrix proteins. The NM-IF Scaffold was dissolved in disassembly buffer (8 M urea, 20 mM MES, 1 mM EGTA, 0.2 mM PMSF, 0.1 mM $MgCl_2$, 1% β ME and pH 6.6 [Bidwell et al., 1993], and then dialyzed against assembly buffer (0.15 M KCl, 2.5 mM imidazole hydrochloride, 5 mM $MgCl_2$, 2 mM DTT, 0.125 mM EGTA, 0.2 mM PMSF, and pH 7.1) for 12 h. The dialysate was centrifuged at 100,000*g* for 95 min to separate soluble NM proteins from the intermediate sized filaments. The soluble NM proteins were further concentrated using a centricon concentrator. The amount of protein in the soluble NM was determined by Bradford analysis [Bradford, 1976].

Gel Electrophoresis

NM proteins were electrophoretically separated on 10% polyacrylamide gels containing 0.1% SDS, and by two-dimensional nonequilibrium gel electrophoresis according to O'Farrell, 1975. The isoelectric focusing gels contained ampholines (BioRad) in the pH ranges 3.5–10 and 6–8, mixed in a ratio of 3:5. Both SDS-PAGE and 2D gels were stained with Coomassie blue.

Immunocytochemical Studies

We raised rabbit polyclonal antibodies against the NM-60 kDa double protein, and its specificity was confirmed by immunoblot (Fig. 2). The αTN-4 cells grown on coverslips in DMEM media containing 20% fetal calf serum, and separated nuclei were washed twice with phosphate buffered saline (PBS) and then fixed with PBS containing 4% paraformaldehyde on ice for 20 min. The fixed cells were incubated with rabbit polyclonal antibodies raised against αTN4 NM-60 (1:100) at 37°C for 1 h. Cells were then washed with PBS, and exposed to goat anti-rabbit IgG fluorescein conjugated antibody (1:100) at 37°C for 30 min. The cells and nuclei were washed with PBS and viewed under a fluorescence microscope.

Amino Acid Sequence Determination

The αTN4 nuclear matrix proteins were separated by 10% SDS-PAGE and then electrophoretically transferred to PVDF paper. The PVDF paper was stained with Coomassie blue. Stained bands of NM-60 were cut from the PVDF paper and trypsinized. Trypsinized proteins were separated by reverse phase HPLC. The internal sequence of NM-60 was determined by automated Edman degradation on an applied Biosystems gas phase sequencer. The sequenced data was analyzed by the BLAST technique [Altschul et al., 1990].

RESULTS

The NM protein of tissue cultured lens epithelial cells was isolated by the modified Penman technique [Bagchi et al., 1995]. Aliquots of fractions isolated at each step of the procedure were collected and analyzed by SDS-PAGE.

Figure 1 displays the protein profile of each extraction step starting with cytoplasmic soluble protein (CS), to the final soluble NM protein. The cell nuclei and adherent intermediate filaments were obtained by hypotonic shock, and the soluble proteins (RS) were discarded. The nuclear fraction (RP) was digested with RNase and DNase to remove nucleic acids and the protein fraction was precipitated with ammonium sulfate (D). The precipitated protein were dissolved in 8 M urea containing buffer, and intermediate filaments (NMP) and soluble nuclear matrix protein were obtained by high speed configuration. The NMP fraction contains mostly intermediate filaments and some low molecular weight protein. A prominent component in the soluble nuclear matrix fraction is a doublet of 60 kDa molecular weight.

Figure 2 shows the protein profile of the soluble NM fraction after 30-fold concentration. The 60 kDa doublet is a prominent component. The low molecular weight protein below 20 kDa may be breakdown products of

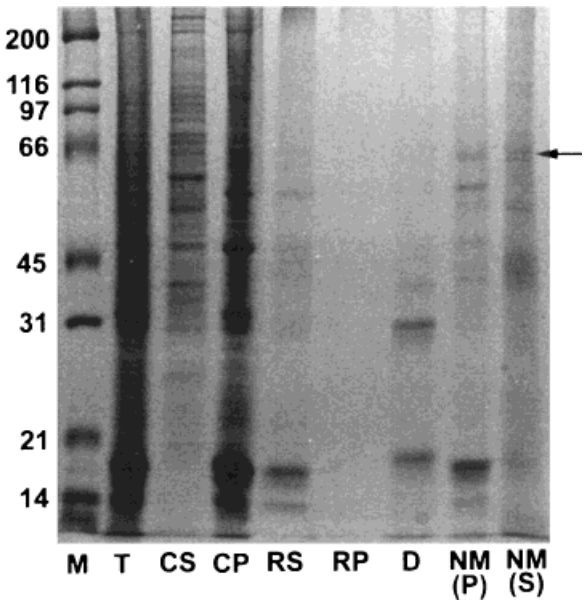

Fig. 1. SDS-PAGE profiles of protein fractions obtained in the isolation procedure of soluble NM proteins of αTN4 cells. T, profile of αTN4 cells dissolved in SDS-buffer; CS, soluble fraction of CSK buffer; CP, pellet of CSK buffer extraction; RS, soluble fraction of RSB – majik extraction; RP, pellet after RSB – majik extraction; D, soluble fraction of digestion buffer extraction; NM (p), pellet after urea extraction; NM(s), soluble nuclear matrix protein, M, molecular weight markers (Bio-rad). Arrow denotes NM-60 protein doublet.

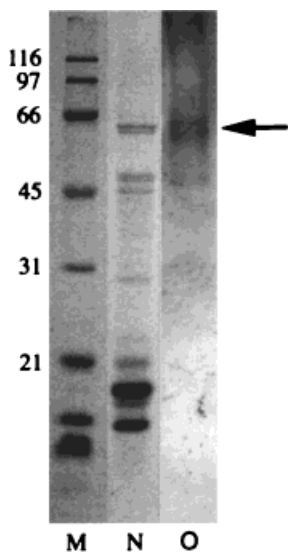

Fig. 2. SDS-PAGE profile of NM(s) protein. M, molecular weight markers; N, NM(s). Arrow denotes NM-60 protein doublet. 0, Immunoblot of N, using anti-NM-60.

higher molecular weight NM protein. Lane '0' depicts immunoblot using anti-NM-60 IgG.

Figure 3 depicts the nonequilibrium 2-D gel electrophoresis profile of soluble NM protein. It demonstrates that the 60 kDa proteins are

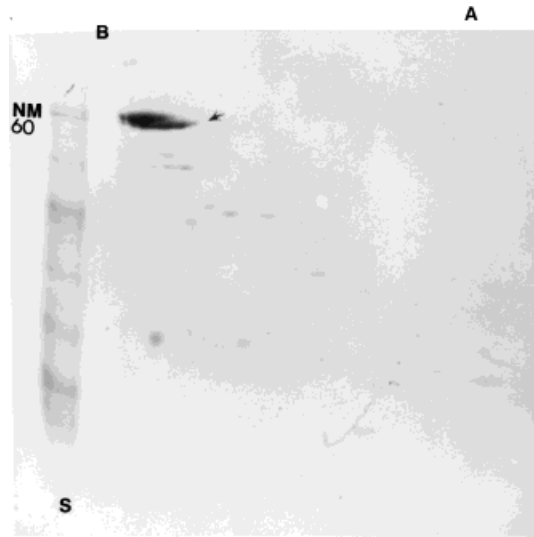

Fig. 3. Nonequilibrium 2D gel electrophoresis of αTN4 NM(s). S, 1-D pattern of NM(s); B, basic area of the gel; A, acidic area of gel. Arrows points to the NM-60 proteins.

highly basic. The identity of the 60 kDa NM proteins were established by immunoblot (data not presented) and by their molecular weight. The regular 2D (IEF plus PAGE) profile showed mostly 45 kDa protein with pH of 5.5, but did not contain the 60 kDa protein, indicating that this protein may be too basic to enter the regular isoelectric focusing gels (pH 4–7.5).

Figure 4 shows that isolated αTN-4 cell nuclei are decorated with FITC- bound NM-60 antibodies. The immunofluorescence results clearly demonstrate that NM-60 is a specific nuclear protein and is located only in the nucleus as there is no fluorescence present in the cytoplasm. The speckled appearance of NM-60 in the nucleus suggests that this protein is present in the discrete areas of the nucleus.

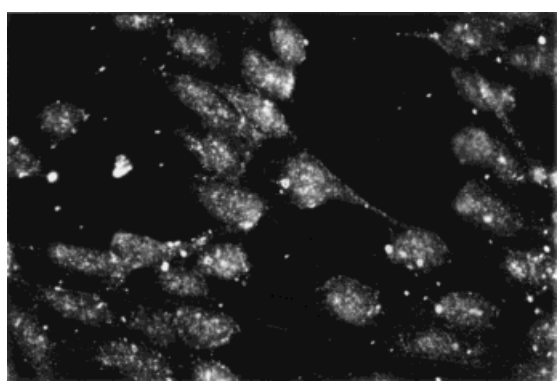

Fig. 4. αTN4 cell nuclei decorated with NM-60 antibody.

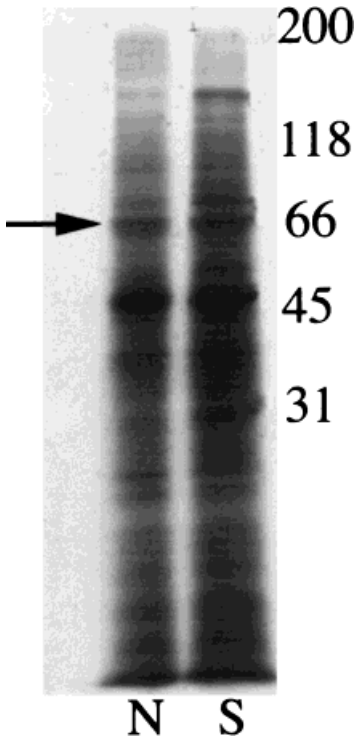

Fig. 5. Autoradiograph of αTN4 NM(s). Twenty micrograms of each protein fraction was applied to the gel; N, αTN4 cells incubated in serum free media; S, cells cultured in 20% serum containing medium.

Figure 5 displays the autoradiogram of NM protein incubated in the presence and absence of 20% fetal calf serum. The autoradiogram shows that many nuclear proteins were synthesized at a higher rate in the αTN-4 cells cultured in serum containing medium. The 60 kDa NM protein of αTN-4 cells incubated in serum containing medium shows a higher rate of synthesis, indicating that serum in the culture medium upregulates the expression of NM-60.

To further characterize the NM-60 proteins, we examined their amino acid sequence by automated applied science amino acid sequences. NM-60 proteins was N-terminally blocked. Thus, trypsin digested internal peptides were sequenced. Three internal peptide fractions sequenced by automated Edman degradation [Heinemann and Ozols, 1998] showed the following composition.

NM-60 Peptide

I	V N L D E N Q N L L N G
II	T G N L E S Y F E V F I N N L
III	G L E I L K N D K

The identity of all three peptide amino acid sequences was examined using the BLAST sequence similarity search [Altschul et al., 1990]. Table I shows that the first peptide (NM-60 PI) has a 75% amino acid sequence similarity with Xenopus laevis heat shock transcription factor 1 (XHSF-1) and a 40% sequence similarity with human, mouse and chicken HSF-1. The sequence homology of NM-60 and HSF-1 predicts that NM-60 Peptide I is probably a carboxy terminal peptide.

NM-60 peptide II (Table II) displays a 40% sequence similarity with *E. coli* RNA polymerase Sigma 32 initiation factor, and NM-60 peptide III shows a 60% sequence similarity with the DnaJ domain of a mosquito protein.

DISCUSSION

This investigation was initiated to examine the nuclear matrix associated protein of tissue cultured αTN-4 cells. The SDS-PAGE profile of the NM fraction showed a 60 kDa doublet band as a prominent protein, and was further characterized. We raised polyclonal antibodies for further characterization of NM-60. Molecular weight was determined by use of molecular weight markers and this protein was further purified by 2-D gel electrophoresis. Regular 2D gel electrophoresis followed by immunoblot failed to localize the 60 kDa protein; however, nonequilibrium 2D gel electrophoresis resolved NM-60 and its identity was confirmed by immunoblot.

To ascertain the specific cellular localization of NM-60, we employed immunofluorescent techniques; and the results clearly demonstrated NM-60 to be a nuclear protein. Furthermore its speckled appearance in the nucleus, suggests that NM-60 is present in the discrete areas of the nucleus. We could not detect any fluorescence in the cytoplasm. To further characterize NM-60, we examined its internal peptide sequence for sequence similarity with known amino acid sequences using BLAST technique [Altschul et al., 1990]. We obtained amino acid sequence of three internal peptide fragments, and searched for their sequence similarity. Data obtained from these searches revealed that all the peptide fragment amino acid sequence show similarity with heat shock transcription factor 1 or heat shock protein. NM-60 PI showed 75% sequence similarity

TABLE I. NM-60 Amino Acid Sequence Compared to Heat Shock Factor (1) Amino Acid Sequences (#395 to 406 Denotes the Location of the Amino Acids in the HSF-1 Protein)

Mouse	NM-60($^{P-1}$)V	N L D E N Q N L L N G	
Xenopus	XHSF-1^{395}F	N L D T L Q N L L N G	406
Human	HHSF-1^{395}S	N L D N Q T M L L S S	406
Mouse	MHSF-1^{395}S	N L D N R T M L L T S	406
Chicken	CHSF-1^{395}S	N L D N Q T M L L S T	406

TABLE II. Amino Acid Sequence Comparison of Two NM-60 Protein Peptide Fraction

NM-60P-2	—	T	G N L E S Y F E V F I N N L	
Sigma-32-		^{13}V	G N L E S Y I R A A N A W P	27
NM-60 P-3	—		G L E I L K N D K	
DnaJ-			+ L E I + K N + K	

with the xenopus heat shock transcription factor (XHSF-1) and about 40% sequence similarities with human/mouse and chicken heat shock factor proteins. NM-60 P2 amino acid sequence displayed 40% sequence similarity with RNA polymerase Sigma-32 initiation factor of *E. coli*. In *E. coli*, the heat shock protein expression is under direct control of Sigma-32 [Narberhaus et al., 1996]. Exposure of *E. coli* to elevated temperature induces synthesis of Sigma-32 and induction of bacterial heat shock protein DnaK (HSP-70 analog) or DnaJ (HSP-40 analog) or GrPE [Silver and Way, 1993]. It has been clearly demonstrated that the levels and activities of Sigma-32 are rate limiting for bacterial heat shock protein gene expression. NM-60 P3 showed 66% sequence similarity with a mosquito protein with a DnaJ domain [Blumberg and Silver, 1991]. Silberstein et al. [1998] reported that many members of HSP-70 family are regulated by co-factors that contain domains homologous to DnaJ. Eucaryotic DnaJ homologues (HSP-40) stimulate the intrinsic ATPase activities of HSP-70, thus activating the molecular chaperone properties of HSP-70. Thus, the amino acid sequence data of NM-60 suggest that it may be related to the regulation of heat shock protein gene expression.

The αTN-4 cells when incubated with 20% fetal calf serum manifest increased α-crystallin synthesis [Yamada et al., 1990]. Experiments were performed to determine whether any specific nuclear matrix protein(s) display correspondingly elevated rates of synthesis in response to fetal calf serum. The data obtained from these experiments showed increased synthesis of several NM proteins, including NM-60 (Fig. 5). Whether the observed higher rate of NM synthesis is related to the increased α-crystallin synthesis needs to be further investigated. It has been reported that increased heat shock protein activity is needed during protein synthesis for the proper folding of newly translated proteins. Thus one can expect elevated synthesis of NM-60, a protein with extensive sequence similarity with heat shock transcription factor 1.

Many of the heat shock proteins are molecular chaperones, which are essential for maintenance of the structural integrity of proteins. It has been demonstrated that the supra molecular organization of crystallins is responsible for lens clarity, and their disturbance would lead to opacification. Thus, lens clarity depends on the fidelity of its structural proteins. Recent studies, indicate that the native state of lens proteins is maintained by the chaperone like activity of α-crystallin and possibly the heat shock proteins HSP-90, HSP-70, and HSP-40.

In all eucaryotes, heat shock protein synthesis is stimulated via sequence specific binding of heat shock transcription factor. Numerous transcription factors have been isolated from NM of different cells [Bagchi et al., 1998], which partition differently between the nuclear matrix (high salt and DNase resistant) and the soluble high salt extractable components of the nucleus. Thus, specific mechanisms exist to regulate the exchange of transcription factors between the NM and the soluble compartment

of the nucleus, which in turn may regulate specific gene expression.

At present it is not known, whether NM-60 is a specific heat shock like transcription factor, which resides in the nucleus to modulate stress response in lens cells or a nuclear matrix structural protein. We are in the process of cloning the NM-60 gene, so that we will have molecular probes to examine the role of this prominent nuclear matrix protein in the regulation of lens cell nuclear function.

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