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Cloning of cDNA encoding a 100 kDa nucleolar protein (nucleoline) of Chinese hamster ovary cells

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ABSTRACT

Nucleoline (100 kDa) is the major nucleolar protein in exponentially growing cells that behaves like a nucleolar organizer protein and plays a key role in rDNA transcription and prerRNA processing. We reported the isolation of 5 cDNA clones by probing a cDNA library, constructed in the expression vector λ gt11, with a polyclonal serum raised against nucleoline. A new immunoassay, using hybrid proteins (βgal-cDNA encoded protein) was developped to establish that the isolated cDNAs encoded parts of nucleoline. A further confirmation resulted from the sequence comparison between the cDNA encoded peptide and a 42 aa peptide isolated from rat nucleoline (1). The 5 cDNAs overlapped extensively and covered more than 90 % of a full length cDNA. By probing a Northern blot with the 100kDa cDNA, a 2650 nucleotide polyA$^+$ RNA was detected that contained just enough information to code for nucleoline.

INTRODUCTION

Ribosome biogenesis is a complex process that requires the expression of more than 100 genes. Most of them encoded products that are directly incorporated in ribosome structure (rRNA and ribosomal proteins). The primary structure of rDNA and 5S genes has been established and sequences needed for their accurate transcription characterized (2-9). Among genes that encoded the 85 ribosomal proteins several have been isolated in different cell species (3, 10). They are present unlinked in the genome as single or few copies (11-14). Another class of proteins plays a key role in ribosome biogenesis : proteins that were localized in the nucleolus and that directly interacted with rDNA or with preribosomes (15-17). Newly synthesized prerRNA becomes immediately associated with ribosomal proteins (18) and with proteins transiently bound to RNA and that remain in the nucleolus (16). Among them a 100 kDa nucleolar protein is the major nucleolar protein in exponentially growing cell and is in amount directly related to the level of rRNA synthesis (19). Its physicochemical characteristics suggest that it is similar to the nucleolar protein C_{23} of Novikoff hepa-

toma cells (1, 20). The 100 kDa protein (nucleoline) is present in most eukaryotic cells and has been highly conserved during evolution. A serum raised against nucleoline extracted from CHO cells cross reacts with nucleolar protein of other species (18).

Several proteins are immunoaffiliated with nucleoline (19). "In vitro" the 100 kDa protein matures by specific proteolytic cleavage in define species (21). Recently a 130 kDa protein was shown to cross react with the 100 kDa serum but did not behave like a precursor of nucleoline. The 100 kDa binds to rDNA, and in particular with sequences localized in the non-transcribed spacers (22). The 100 kDa has no effect in "in vitro" transcription experiments using the 5'end of rDNA as template. The protein is automatured in defined species during the incubation. However in presence of leupeptin the automaturation is blocked and the 100 kDa acts as an inhibitor of transcription (23). The 100 kDa is also recovered associated with preribosomes and it can play a role in the processing of prerRNA (21). Nucleoline appears as a multifunctional protein that plays a key role in preribosome biogenesis and in nucleolar organization.

In this paper, we report the isolation and characterization of cDNA encoding nucleoline. Cloning has been carried out in the gt11 expression vector. The five isolated clones overlapped extensively and represent more than 90 % of a full length cDNA. Two clones differed in their 5'end and 3'ends and encode two proteins identical on 400 amino acids.

MATERIALS AND METHODS

Materials

CHO cells were grown in the laboratory as described previously (24). *E. coli* strains : Y1004, Y1088, Y1089 and Y1090 were from R. Young (25, 26). They were used respectively to produce λ gt11, to construct and amplify recombinant phages, to produce hybrid proteins and to screen the cDNA library. Labeled nucleotides were from Amersham (England).

cDNA synthesis and cloning in λ gt11

cDNA was synthesized according to a previously described technique (27). EcoRI linkers were then added (0.5 µg linkers / 1 µg cDNA) using T4 DNA ligase. After treatment with EcoRI restriction enzyme, the free linkers were separated from cDNA by chromatography on A50m Biogel. cDNA was precipitated by 2 vol. ethanol. cDNA (25 ng) was ligated to EcoRI cleaved λ gt11 (1 µg). After encapsidation, phages were adsorbed to Y1090. 15 % of the resulting plaque contained recombinants. Screening with antibodies was

carried out as described by Young and Davis (25).

Characterization of DNA inserts and proteins

Lysogens were constructed from recombinant phages using strain Y1089. Lysogens were grown in 10 ml of rich medium. At an 0.6 OD (600 nm) they were incubated for 30 min at 44°C, induced for β-galactosidase by addition of 5 mM IPTG for 90 min at 38°C and collected by centrifugation. The pellet was resuspended in 500 µl of sample buffer (125 mM Tris pH 8.8, 0.1M DTT, 10 % Glycerol, 10 mM EDTA) and boiled for 3 min. After 10 min centrifugation in an Eppendorf microfuge, 30 µl of the supernatant were directly deposited on a 7.5 % polyacrylamide gel (28) and electrophoresis was for 4 h at 30 mA; the gels were then stained with Coomassie blue. An unstained duplicate gel was electrotransferred onto nitrocellulose paper and immunoreacted with the purified anti 100 kDa IgG as previously described (19). Cyanogen bromide cleavages were carried out on polyacrylamide gel slices containing the 100 kDa as described by Pepinsky (29).

To prepare cDNA inserts, recombinant phages were purified from 1 l of a lytic culture (Y1088) and phage DNA was extracted (30). Purified DNA was digested with EcoRI and the fragments were separated by electrophoresis on a 0.8 % agarose gel. Inserts were purified and after labeling by nick-translation, used to screen a Northern blot of total polyA$^+$ RNA (31). Fractionation by electrophoresis, blotting, immunodetection of the 100 kDa protein were carried out as previously described (19).

RESULTS

Cloning of the 100 kDa cDNA

The 100 kDa nucleolar protein is involved in the synthesis and maturation of prerRNA. Its cellular concentration is directly related to ribosome production (19). Exponentially growing CHO cells in which the 100 kDa protein represents around 0.1 % of total cellular protein, were used as a source of mRNA to construct a cDNA library. cDNAs were cloned in the EcoRI site of the λgt11 expression phage. The cloning site lies in the β-galactosidase gene (25).

LacZ$^-$ bacteria plated on a medium containing IPTG gave respectively blue and white plaques when infected by the wild λgt11 or recombinant phages. The recombinants that produced hybrid proteins were screened with a serum raised against the 100 kDa protein that has been purified by chromatography through a Sepharose 4B column to which was bound a crude protein extract of Y1089 strain lyzogenized by λgt11. 5 positive clones were ob-

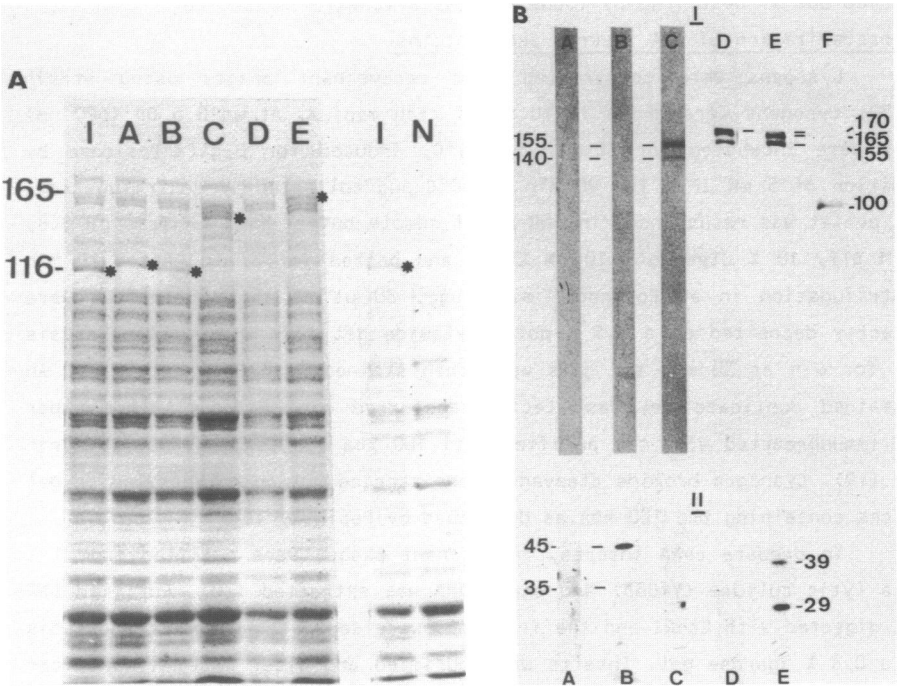

Figure 1 : Characterization of λ gt11 β-galactosidase - fusion protein.
A : Lysogens were constructed in the Y1089 strain with the 5 different λ gt11 clones that gave a strong signal with the anti 100 kDa serum. Bacterial lysates were prepared from the lysogens thus obtained following incubation at 42°C to inactivate the temperature sensitive repressor and incubation in presence of IPTG to induce the lacZ transcription. After centrifugation to remove bacterial fragments, samples were analyzed on a 7.5 per cent polyacrylamide gel and electrophoresis was run for 4 hours. Gel was then Coomassie blue stained.
B : Proteins were electrotransferred from the polyacrylamide gels to nitrocellulose filters. Immunodetection was carried out with the anti 100 kDa antibodies and ^{125}I protein A (18). (I) : 7.5 % polyacrylamide gel. (II) : 12 % polyacrylamide gel. I and N : λ gt11 without cDNA insert. I : IPTG induced. N : uninduced. A-E : 5 different induced recombinants. A : BL02.1; B : BL15.1; C : BL16.1; D : BL16.2; E : BL17.1. (*) : IPTG induced proteins; F : 100 kDa run as control (0.1 μg).

tained out of 1.5 10^4 screened recombinants (27).

To demonstrate that the selected recombinants contained cDNA encoding the 100 kDa, characterization of the hybrid proteins was carried out. The five clones were grown in presence of IPTG and cellular extracts were directly analysed by gel electrophoresis. Proteins were visualized directly by Coomassie blue staining of the gel or after transfer to nitrocellulose

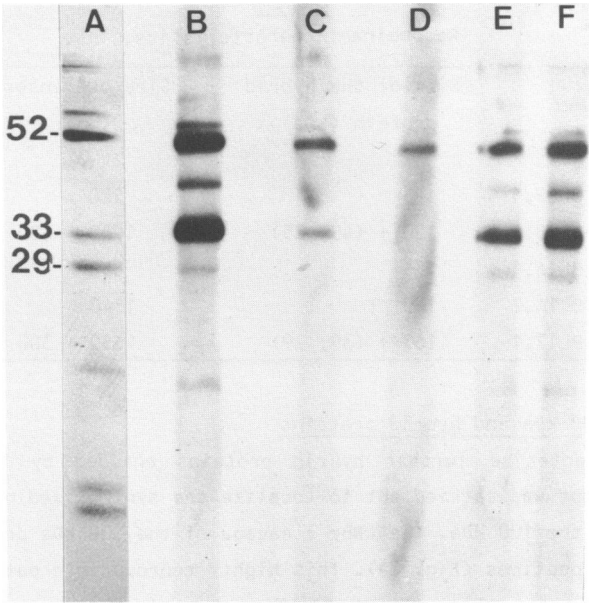

Figure 2 : Immunocompetition between 100 kDa CNBr peptides and hybrid proteins.

Identical polyacrylamide gel slices containing 10 µg of 100 kDa protein were excised from a slab gel and exposed to CNBr (29). At the end of the treatment and after extensive washing, gel strips were immediately electrophoresed into SDS-polyacrylamide gels (28). Gel was Coomassie blue stained (A), or the corresponding Western blot was probed with antibodies (B-F). The 100 kDa serum was immunodepleted by running on Sepharose 4B column on which was bound protein extracts from lysogens that have been constructed in Y1089 rising BL16.2 or BL17.1 recombinant λ gt11 phages (26).Two IgG fractions were obtained : bound and unbound. Probing was carried out with : B : unfractionated IgG; C : BL16.2 unbound; D : BL17.1 unbound; E : BL16.2 bound; F : 17.1 bound.

filter by probing with purified anti 100 kDa antibodies then by reaction with ^{125}I protein A. As shown in figure 1, the 5 clones gave different signals. In BL16.1 and BL17.1 the number of chimeric molecules is high enough to allow a direct visualization of the induced species by Coomassie blue staining of the gel while in BL15.1 and BL16.2 hybrid proteins were only visualized by immunodetection. Thus, in four cases, the proteins encoded by the cDNA can be detected by the antibodies raised against nucleoline. It must be noted large variation in the production of hybrid protein by different clones that contained more than 70 % overlapping cDNA inserts (compare BL16.2 and BL17.1).

TABLE 1

Recombinant characteristics.

	Size of the hybrid protein (kDa)	Size DNA insert (bp)
BL02.1	125	520
BL15.1	120 + (45; 35)	1980 + 480
BL16.1	155	850
BL16.2	170	1840
BL17.1	165 + (39; 29)	1650 + 300

Structure of 100 kDa and hybrid proteins

To characterize further hybrid proteins encoded by the selected cDNAs, an attempt was carried out to localize the synthesized proteins on a peptide map of the 100 kDa. The CNBr cleavage of the 100 kDa protein resulted in 7 major peptides (Fig. 2A). This highly reproducible pattern contained partial digests, that can be further cleaved only when the purified fragments were treated again by CNBr. The 7 peptides were differently recognized by the polyclonal 100 kDa serum (Fig. 2B). After Western blotting, 2 peptides (52 kDa and 33 kDa) presented a strong immunoreactivity. The 29 kDa peptide, that contained most of the phosphate groups, is localized in the NH_2 part of the molecule and reacts only slightly.

The Western blot of the 100 kDa CNBr peptides was probed with a serum depleted in immunoglobulin that recognized the BL16.2 and BL17.1 hybrid proteins. The serum was run on a Sepharose 4B column to which were bound crude protein extracts of Y1089 strain lysogenized by λgt11 recombinant containing the BL16.2 or the BL17.1 insert. As shown in Fig. 2 (C-D) the BL17.1 depleted serum recognized only the 52 kDa peptide but the signal was lower than with undepleted serums. The BL16.2 depleted serum gave a slight residual signal with the 52 kDa and 31 kDa. The antibodies that were bound on E. coli Sepharose 4B column were eluted and used to screen Western blots of 100 kDa CNBr digests. All the peptides that were not recognized by the depleted serums gave again strong signals. These results demonstrate that the cloned cDNAs encode large part of the 100 kDa protein and in particular the antigenic area which is consistent with the technique used for the screening of the cDNA library.

Alignement of 100 kDa cDNA clones

DNAs from the 5 different clones were purified and analyzed by res-

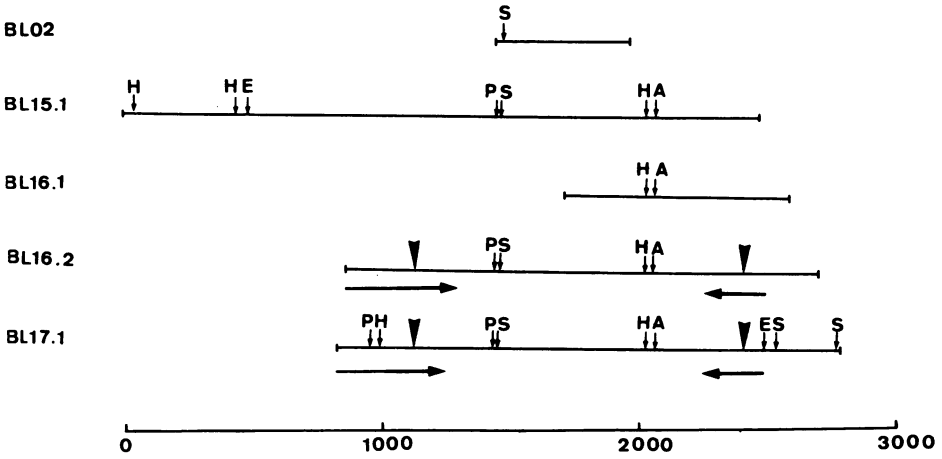

Figure 3 : Alignement of cDNA clones.
Five clones were aligned by comparison of restriction maps of the cDNAs inserts. Restriction sites for AvaII (A), EcoRI (E), HindIII (H), PvuII (P), SstI (S) are marked. Between positions 1050 and 2250 maps were concordant. Divergences in BL17.1 5'and 3'ends were determined by sequencing (⟶) and are marked by arrowheads (▼).

triction mapping. To determine the orientation of the coding sequence, restriction maps of the recombinant phage DNA were carried out using SstI and KpnI restriction enzymes. BL15.1 and BL17.1 inserts contained an EcoRI site (Table 1). The small fragments generated by the EcoRI digestion were respectively localized at the 5'end and 3'end of BL15.1 and BL17.1. The 3 large inserts possessed a complete homology on 1200 nucleotides. However in

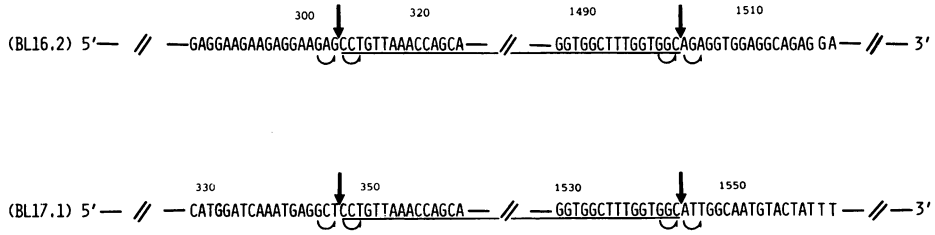

Figure 4 : Comparison of BL16.2 and BL17.1 sequences.
Fragments of BL16.2 and 17.1 were cloned in Mp10 and Mp11 and were sequenced according to Sanger (34), (↓) : junction between conserved and divergent sequences. The underlined 1197 nucleotides are similar in BL16.2 and BL17.1 (——). (◡) : reading frame.

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                                                                    90
16.2 : AAG GTA GCT GCT GCT GCT CCT GCC TCA GAG GAT GAA GAC GAG GAA GAG GAT GAA GAG GAG
       LYS VAL ALA ALA ALA ALA PRO ALA SER GLU ASP GLU ASP GLU GLU GLU ASP GLU GLU GLU
C23; CA :    ala ala pro ala ala pro ala ser glu asp glu asp glu glu asp asp asp glu asp
                                                                   150
16.2 : GAG GAG GAG GAT GAA GAG GAG GAG GAT GAC TCT GAA GAA GAA GAA GCT ATG GAG ATC ACA
       GLU GLU GLU ASP GLU GLU GLU GLU ASP ASP SER GLU GLU GLU GLU ALA MET GLU ILE THR
       asp asp asp asp ser gln glu ser glu glu ser glu asp glu glu val met glu ile thr
                                                                   210
16.2 : CCT GCC AAA GGA AAG AAA GCT CCT GCA AAA GTT GTT CCT GTG AAA GCC AAG AAT GTG GCT
       PRO ALA LYS GLY LYS LYS ALA PRO ALA LYS VAL VAL PRO VAL LYS ALA LYS ASN VAL ALA
C23; CA  pro ala lys
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Figure 5 : Sequence comparison of the protein encoded by BL16.2 and the peptide CA of protein C_{23}.
The 5'end of BL16.2 was sequenced on 160 nucleotides and traducted in aminoacid sequence. The sequence of the resulting peptide is compared with the published sequence of the peptide CA of protein C_{23} (1).

the vicinity of both ends, BL17.1 cDNA contained two restriction enzyme sites (PvuII and HindIII) that were not present in BL16.2 (Figure 3). After cloning in M13, BL16.2 and BL17.1 were sequenced. No sequence homology is observed at both ends. The common 1200 nucleotide stretch starts respectively at positions 304 (BL16.2) and 346 (BL17.1) (Figure 4).

Comparison of BL16.2 encoded protein and C_{23} protein sequences

In figure 5 is shown the nucleotidic sequence of the BL16.2 5'end and its traduction in amino acids. The corresponding peptide is particularly acidic since on 50 aminoacid it contains 26 glu or asp. Twenty five are clustered on a continuous stretch. A peptide with an analogous composition was found in the rat protein C_{23} (19). It contained 23 glu and asp, twenty two being in a cluster. There is only 64 % homologies between rat and hamster but most of the divergences result from replacement of glu by asp and vice versa. This is achieved by a single change in the third nucleotide of the codons. Outside the glu-asp stretch a 90 % homologie is observed. The sequence of another peptide of protein C_{23} (CB5 : 43 aa) is recovered in the protein encoded by BL16.2 with a 100 % conservation (Lapeyre et al., submitted for publication).

Characterization mRNA encoding the 100 kDa protein

PolyA$^+$ mRNA extracted from exponentially growing CHO cells were sized by electrophoresis on a methyl mercuric agarose gels. RNA was then electroblotted on a DBM paper. Northern blots were probed with BL16.2 cDNA labeled by nick-translation. As shown in figure 6, a 2650 nucleotides major band was detected. Two other species hybridized at a lower extent, while a

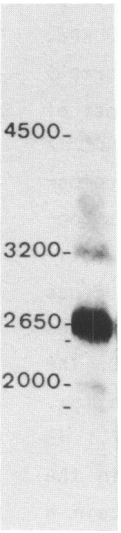

Figure 6 : Characterization of nucleoline mRNA.
Total polyA$^+$ mRNA was run in 0.8 % agarose gel containing 20 mM methylmercuric hydroxide. The corresponding Northern blot was probed with the BL16.2 insert that has been labeled by nick-translation. Ribosomal markers : 18S (1879 nt); 28S (4713 nt).

faint band (4500 nt) can be visualized after long exposure time. Taking into account the presence in polyA$^+$ mRNA of 250 untranslated nucleotides it can be calculated that the 100 kDa contained 800 aminoacids. According to the 100 kDa amino acid composition (32), the average molecular weight of amino acid is 124 and the calculated molecular weight of the protein is 99.000.

DISCUSSION

The isolation and the characterization of cDNA encoding the 100 kDa nucleolar protein in the λ gt11 expression vector conduct to significant new results on the origin of protein immunoaffiliated with nucleoline. In particular, the size of the 100 kDa mRNA ruled out the existence of precursor to product relationships between the two immunologically related 130 kDa and 100 kDa nucleolar proteins (33).

The procedure described in figure 2 appeared particularly suitable to demonstrate that the selected cDNAs encoded the 100 kDa protein. The 100 kDa serum could contain antibodies raised against a contaminant of the 100 kDa protein. Probing with such serum a cDNA library could conduct to the isolation of cDNA encoding the contaminant protein. However the CNBr cleavage will produce different peptide pattern for nucleoline and the contaminant. Fractionation of the serum in antibodies that unbound or bound to the hybrid proteins allows to detect unambiguously on Western blots the

peptides that are both present in the 100 kDa and in the hybrid proteins. The results demonstrate that the isolated cDNAs encode most of the 100 kDa. Concomitently we have shown that the antigenic sites are not uniformely distributed along the molecule. The NH_2 terminal part that contains most of the phosphate moieties (32) was only poorly recognized by the serum. A further demonstration that BL16.2 encodes nucleoline was achieved by comparison of the translated sequence with the sequences of five peptides contained in the protein C_{23}. We have previously shown that monoclonal antibodies raised against the rat protein C_{23} (gift of Dr. H. Busch) cross reacted with the hamster 100 kDa protein. The sequences of four peptides (30 TO 40 aa each) are recovered in the protein encoded by BL16.2. The unrecovered peptide corresponds to the NH_2 terminal end of protein C_{23}. BL17.1 does not encode the peptide shown in figure 5. However BL17.1 is read in frame with B-galactosidase at the 5'end and with BL16.2 in the conserved DNA stretch. Thus the proteins (X) encoded by BL17.1 contain a peptide (400 aa) recovered in the 100 kDa but is different from nucleoline. This common peptide is immunogene (Figure 2 and 3) and (X) must be one of the nucleolar proteins immunoprecipitated by the serum anti 100 kDa (18). Northern blot hybridization with BL16.2 also demonstrated the existence of several mRNAs. The major species was 2650 nucleotides and contained just enough informations to encode the 100 kDa taking into account the 100 kDa amino acid composition (32). Two species (3200 and 4500 nucleotides) could code for 130 kDa and 125 kDa phosphorylated nucleolar protein that cross reacted with the anti 100 kDa serum (33). Peptide map of these two proteins confirmed sequence homologies with the 100 kDa. Preliminary Southern blotting data using CHO genomic DNA and BL16.2 probe suggest that there is only one gene for the 100 kDa protein. Furthermore the number of mRNA that hybridized with BL16.2 cDNA is lower than the number of proteins recognized by the serum anti 100 kDa. Thus the family of proteins that are immunologically related to nucleoline could result from two different processes : differential splicing of a same premRNA (at least to produce the 130 kDa and 100 kDa proteins) and maturation of the 100 kDa protein.

The sequence determination of the different cDNAs will allow to determine the primary structure of the various proteins, the mode of differential premRNA splicing and of protein maturation. The complete characterization of the multifunctional 100 kDa protein is the first step in the knowledge of nucleolar organization.

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