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Molecular cloning of cDNA for the nuclear ribonucleoprotein particle C proteins: A conserved gene family

(heterogeneous nuclear RNA packaging/RNA splicing/monoclonal antibodies/λgt11 expression vector)

TERRY Y. NAKAGAWA*, MAURICE S. SWANSON*, BARBARA J. WOLD†, AND GIDEON DREYFUSS*§

*Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60201; and †Division of Biology, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **The C proteins, C1 and C2, are major constituents of the heterogeneous nuclear RNA (hnRNA) ribonucleoprotein (hnRNP) complex in vertebrates. C1 and C2 are antigenically related phosphoproteins that are in contact with hnRNA in intact cells and bind to RNA tightly *in vitro*. A cDNA clone for the C proteins was isolated by immunological screening of a human λgt11 expression vector cDNA library with monoclonal antibodies. The *lacZ*-cDNA fusion protein is recognized by two different anti-C protein monoclonal antibodies. HeLa cell mRNA that was hybrid-selected with the cDNA clone (1.1 kilobases long) was translated *in vitro* and yielded both the C1 and C2 proteins (41 and 43 kDa, respectively). RNA blot analysis showed strong hybridization to two polyadenylylated transcripts, of about 1.4 kb and 1.9 kb, in human cells. Genomic DNA blot analysis showed multiple hybridizing restriction fragments in human and mouse, and homologous DNA sequences are found across eukaryotes from human to yeast. These findings suggest that the sequences encoding the hnRNP C proteins are members of a conserved gene family and they open the way for detailed molecular and genetic studies of these proteins.**

In eukaryotic cells, the primary nuclear transcripts of RNA polymerase II, some of which are precursors to cytoplasmic mRNA (pre-mRNA), are collectively referred to as heterogeneous nuclear RNAs (hnRNAs). Considerable biochemical (1–21) and microscopic (22–24) evidence indicates that the hnRNAs are found in the nucleus in association with a specific set of proteins to form ribonucleoprotein (hnRNP) particles. The vertebrate C proteins, C1 and C2, are major constituents of these particles and are components of the 30S complexes, which are monomers of the hnRNP particles (refs. 4, 14, 15, 21, 25; G.D. and Y. D. Choi, unpublished data). These abundant nucleoplasmic proteins are in contact with the hnRNA *in vivo* and are efficiently crosslinked to it upon UV irradiation of intact cells (18, 20, 21, 26). The tight association of the C proteins with the hnRNA is also reflected in isolated hnRNP complexes by their relative resistance to dissociation from the hnRNA at high salt concentrations (4, 15); whereas, for example, the A and B hnRNP proteins dissociate from the hnRNA at salt concentrations of 200–300 mM NaCl, the C proteins are dissociated only at about 750 mM NaCl (4, 15). By use of monoclonal antibodies, the C proteins were identified in widely divergent vertebrates from human to lizard (20, 26). In all species examined, there are two C proteins in the molecular weight range of 39,000–42,000 for C1 and 40,000–45,000 for C2. The two C proteins are highly related to each other immunologically and both are extensively phosphorylated and have the same isoelectric point (pI = 6.0 ± 0.5) (20, 26).

The precise relationship between C1 and C2 and between the C proteins and other RNP proteins remains unclear. The two C protein bands in $NaDodSO_4$/polyacrylamide gel electrophoresis are not likely to arise from the same polypeptide by posttranslational modification, because their ratio is always the same after any biochemical and cellular fractionation so far attempted. Further, the same C1/C2 ratio as is found in cells is seen after *in vitro* translation of HeLa mRNA in rabbit reticulocyte lysates and immunoprecipitation, suggesting that they are likely to be translated from two different mRNAs (N. Theodorakis and G.D., unpublished results). The possibility has been raised that other hnRNP proteins related to C1 and C2 exist (27). However, in the absence of amino acid sequence data, these issues cannot yet be addressed.

Because of their abundance and tight association with the hnRNA, it seems likely that the C proteins play a central role in the formation of the ubiquitous hnRNP complex. Furthermore, the C proteins occupy peripheral and hnRNA-contacting positions in the 30S hnRNP monoparticle (refs. 20, 21, 25, 26; G.D. and Y. D. Choi, unpublished data) and may therefore be important not only in the packaging of the hnRNA in the nucleus but also in the processing of pre-mRNA. In fact, recent results show that a monoclonal antibody to the C proteins inhibits the splicing of pre-mRNA *in vitro* and that the C proteins are constituents of the 60S pre-mRNA splicing complex (49).

We have undertaken the isolation of cDNA for the C proteins in order to obtain information about their amino acid sequence, their mRNA(s), and the number, evolution, and organization of their genes. Only very limited information about hnRNP proteins is presently available. We report here the isolation of a cDNA clone for the human C proteins and describe the detection of homologous mRNAs and homologous genomic DNA in eukaryotes from human to yeast.

MATERIALS AND METHODS

Antibodies. The preparation and characterization of the monoclonal antibodies to the C hnRNP protein, 4F4 and 2B12, have been described (20, 26).

Immunologic Screening of the λgt11 Library. The λgt11 cDNA library was prepared from poly(A)$^+$ RNA (28, 29) from simian virus 40 (SV40)-transformed human WI-38 cells essentially as described (30).

The immunobiological screening was carried out essentially as described by Young and Davis (29). λgt11 recombinant phages were plated on *Escherichia coli* Y1090 at 5×10^4 plaque-forming units per 150-mm plate. Expression of β-galactosidase fusion proteins was induced by overlaying the

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Abbreviations: hnRNA, heterogeneous nuclear RNA; hnRNP; heterogeneous nuclear ribonucleoprotein; IPTG, isopropyl β-D-thiogalactoside; kb, kilobase(s).

§To whom correspondence should be addressed.

plates with nitrocellulose filters (BA-85, Schleicher and Schuell) that had been saturated with 10 mM isopropyl β-D-thiogalactoside (IPTG) and air-dried. The filters were placed on the top agar and the plates were incubated upside down for 10–12 hr at 37°C. After the transfer of the induced proteins to the nitrocellulose, the filters were incubated in 5% nonfat dry milk in phosphate-buffered saline (PBS, 137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.4) (26). The monoclonal antibodies were used for screening at 1:1000 (4F4) and 1:5000 (2B12) dilutions; incubations were overnight at room temperature. Unbound antibody was removed by three successive washes with PBS, PBS containing 0.05% Nonidet P-40, and PBS again. To detect specifically bound antibody, the filters were incubated with ^{125}I-labeled goat anti-mouse IgG (2.5×10^5 cpm/ml) for 2–4 hr, washed as above, and exposed to x-ray film for autoradiography (12–18 hr).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis and Immunoblotting. NaDodSO$_4$/PAGE was carried out using the discontinuous system described recently (31). Immunoblotting procedures were essentially according to Burnette (32) as previously detailed (26).

Lysogenization and Preparation of Fusion Proteins. Lysogens were prepared in Y1089 with the aid of λh80MΔ selector phage (33). Lysogenic cells were grown to OD_{600} = 0.4 at 30°C, upon which time lytic replication was induced by a temperature shift (42°C, 15 min). IPTG (10 mM) was added and the culture was incubated for an additional 2 hr at 38°C. Cells were collected by centrifugation and boiled in 1 volume of double-strength NaDodSO$_4$/PAGE sample buffer. Samples were clarified by centrifugation (13,000 × *g*, 10 min) and applied to a NaDodSO$_4$ 10% polyacrylamide gel (31).

Hybrid-Selection and *in Vitro* Translation. Poly(A)$^+$ RNA was prepared from HeLa cell cytoplasm by phenol extraction in the presence of the RNase inhibitor, vanadyl–adenosine (10 mM) and chromatography on oligo(dT)-cellulose (34, 35). Hybridization-selection was performed using a modification of the technique of Ricciardi *et al.* (36). The plasmids pGEM-1 (Promega Biotec, Madison, WI) and pHC4F4 (pGEM-1 bearing the cDNA insert) were digested with *Eco*RI, denatured by both boiling and treatment with 0.5 M NaOH, and applied to nitrocellulose following neutralization. Approximately 50 μg of HeLa cell poly(A)$^+$ RNA was used for each hybridization, which was performed at 50°C for 3 hr in 65% (vol/vol) formamide/20 mM Pipes, pH 6.4/0.4 M NaCl/0.2% NaDodSO$_4$ containing yeast tRNA at 150 μg/ml. After extensive washing at 65°C, specifically bound RNAs were eluted for 15 min at 20°C with 10 mM methylmercuric hydroxide containing tRNA at 10 μg/ml. Dithiothreitol was then added to a final concentration of 20 mM, 0.1 volume of potassium acetate (pH 5.0) was added, and the RNA was precipitated with two volumes of ethanol. Optimized *in vitro* translations, employing rabbit reticulocyte lysates (37), were performed for 60 min at 37°C in 50-μl reaction volumes and analyzed by NaDodSO$_4$/PAGE and fluorography (38).

RNA Blot Hybridization. Poly(A)$^+$ RNA was resolved by electrophoresis in a 1.4% agarose gel in the presence of formaldehyde (39), and the nitrocellulose blot was prepared as described (28). The blot was probed with ^{32}P-labeled RNA produced by transcription *in vitro* of the cDNA insert (*Eco*RI subclone of the λHC4F4 insert in pGEM-1, pHC4F4, linearized with *Bam*HI) with the T7 polymerase according to the instructions of the supplier (Promega Biotec). Hybridizations were carried out, essentially as described by Melton *et al.* (40), at 60°C in buffer containing 50% (vol/vol) formamide. The blot was washed and exposed to an x-ray film for autoradiography.

Southern Blots of Genomic DNA. Mouse (B.10A) (41) and *Drosophila melanogaster* (42) (Oregon R) high molecular weight DNAs were prepared as described previously. Human and yeast DNAs were the kind gifts of R. Morimoto (Northwestern University) and T. Donahue (Northwestern University Medical School), respectively. Southern blot (43) hybridizations for human and mouse genomic DNA were for 24 hr at 42°C in 50% formamide/5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.2% NaDodSO$_4$/5× Denhardt's solution (44) containing calf thymus DNA (100 μg/ml) and poly(A) (1 μg/ml). Blot hybridization for yeast genomic DNA was performed at 50°C in 5× SSPE (1× SSPE = 0.18 M NaCl/0.01 M sodium phosphate, pH 7.4/1 mM EDTA)/0.1% NaDodSO$_4$/5× Denhardt's solution containing calf thymus DNA (200 μg/ml). Blots were washed in 1× SSC/0.1% NaDodSO$_4$ at either 65°C (human and mouse) or 50°C (yeast).

RESULTS

A mixture of two monoclonal antibodies, 4F4 and 2B12, each of which recognizes different antigenic sites on both the C1 and C2 hnRNP proteins (20, 26) was used to screen a λgt11 expression vector library (29) containing cDNA prepared from human fibroblastic cells. Initial plaque-screening of ≈300,000 recombinants identified one putative C-protein clone. Upon several rounds of purification and rescreening, a pure clone was obtained in which every plaque was reactive with both 4F4 and 2B12 but not with nonimmune mouse serum. To demonstrate that the reactivity of the phage clone, λHC4F4, with the antibodies was due to antigenic determinants encoded by the cDNA insert, a lysogen was prepared and tested to determine whether the *lacZ*-cDNA fusion protein bound the antibodies. Protein lysates from the lysogen induced by temperature shift and IPTG were prepared, and the proteins were screened separately with 4F4/2B12 and anti-β-galactosidase antibody by immunoblotting after NaDodSO$_4$/PAGE. Fig. 1 shows that a β-galactosidase-containing hybrid protein of ≈150 kDa is induced which reacts with both 4F4 and 2B12. The cDNA insert, therefore, encodes at least two distinct antigenic determi-

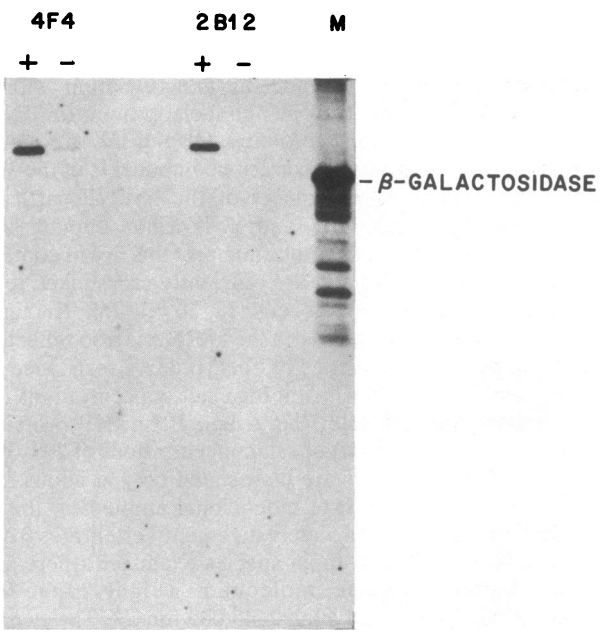

FIG. 1. Characterization of the *lacZ*-cDNA fusion protein. Lysates were prepared from IPTG-induced (+) and uninduced (−) lysogens of λHC4F4 and analyzed by immunoblotting. Immunoblots were probed with the anti-C hnRNP antibodies 4F4 and 2B12. A monoclonal antibody to λ-galactosidase (lane *M*) was used to probe β-galactosidase as a molecular weight marker. Antibody detection was with ^{125}I-labeled goat anti-mouse IgG.

nants that are found in vertebrate cells only on the C1 and C2 proteins (26). Further, immunoblotting of HeLa cell lysate with serum of a mouse that was immunized with this fusion protein detects the C1 and C2 proteins (unpublished results).

The cDNA insert was isolated from the λHC4F4 phage by digestion with *Eco*RI and was further characterized by size and restriction analysis. The cDNA 1.1-kilobase (kb) insert was subcloned into the twin bacteriophage promoter plasmid, pGEM-1, to yield pHC4F4. To confirm the identity of the cDNA clone, pHC4F4 was used for hybrid selection of HeLa mRNA. The hybrid-selected mRNA specifically translated *in vitro* two proteins, of 41 and 43 kDa (Fig. 2, lane C), that were immunoprecipitated with the anti-C proteins monoclonal antibody 4F4 (Fig. 2, lane F). pHC4F4, therefore, harbors a cDNA insert that is highly homologous to the mRNAs of both C1 and C2. This experiment confirms the identification of pHC4F4 and, because of the high stringency of the hybridization, indicates that the mRNAs encoding C1 and C2 are identical or have extensive sequence homology. At this point, we do not know whether pHC4F4 is a partial cDNA clone of C1 or of C2. In the sample hybrid-selected with pHC4F4, there is also increased signal at about 34 kDa (lane C). This may be the result of a specific selection and raises the possibility that the cDNA pHC4F4 also has homology with another, non-C protein. We note that other hnRNP proteins, such as the A proteins, have about that size (4, 21).

To investigate the homologous mRNA, poly(A)$^+$ RNA from HeLa cells was fractionated by electrophoresis in a denaturing formaldehyde/agarose gel, blotted onto nitrocellulose paper, and hybridized with the full-length antisense transcript of pHC4F4. This cDNA transcript hybridizes at high stringency to two major bands at about 1.4 and 1.9 kb (Fig. 3); however, several minor bands at larger size are also detected. Since the

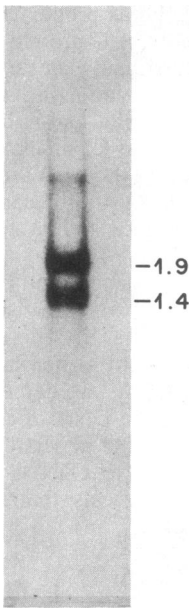

FIG. 3. Blot of electrophoretically separated HeLA mRNA (1.4 μg per lane) probed with the T7 polymerase RNA transcript of the pHC4F4 cDNA insert. pHC4F4 was liberated at the unique *Bam*HI site in the polylinker and used as a template to produce the antisense RNA for the T7 promoter. Sizes of hybridizing bands are given in kb.

C1 and C2 proteins are highly related immunologically but have a difference of mobility in NaDodSO$_4$/PAGE corresponding to about 2 kDa, it is likely that one of the mRNAs corresponds to C2 (43 kDa) and the other to C1 (41 kDa). Since pHC4F4 does not contain a copy of the poly(A) tail of the mRNA as determined by DNA sequencing (data not shown), the cDNA clone in pHC4F4 corresponds to a substantial percentage of the homologous mRNA.

To investigate the genomic complexity of the C-protein genes in mammals, Southern blot analysis was performed. As Fig. 4 illustrates, multiple bands are detected in both human and mouse DNAs by high-stringency hybridization using the

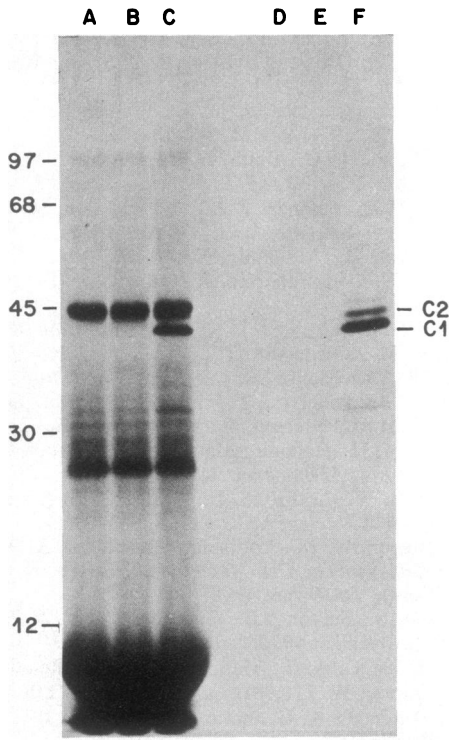

FIG. 2. Hybrid-selection translation of HeLa mRNA with pHC4F4. Hybridization selection and *in vitro* translation of 50 μg of poly(A)$^+$ RNA isolated from log-phase HeLa cells were performed as described in *Materials and Methods*. Lanes: A, nitrocellulose control without added DNA; B, pGEM-1; C, pHC4F4; D–F, immunoprecipitates of material in lanes A–C. Fluorography was for 12 hr. Positions and sizes (kDa) of markers run in parallel are shown at left.

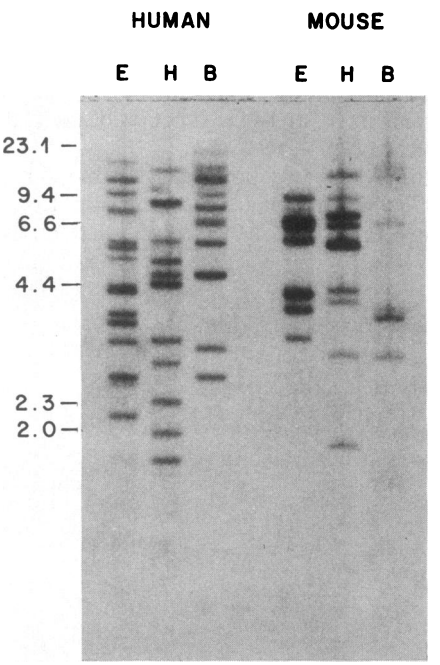

FIG. 4. DNA blot analysis of C-protein genes. Human and mouse genomic DNA (10 μg per lane) was digested with either *Eco*RI (lanes E), *Hin*dIII (lanes H) or *Bam*HI (lanes B), electrophoresed in 1% agarose gel, blotted onto nitrocellulose, and hybridized with the nick-translated *Eco*RI insert of pHC4F4. Hybridization was at 42°C with 50% formamide, as detailed in *Materials and Methods*. Markers (kb) at left represent positions of *Hin*dIII restriction fragments of λ phage DNA.

nick-translated *Eco*RI insert of pHC4F4 as probe. Some of these bands may result from fragments of C-protein genes, but it is also possible that multiple functional genes exist for the C proteins or that some of the hybridizing species represent pseudogenes. Since the RNA blot and hybridization-selection analyses detect both C1 and C2 mRNAs, it is likely, given the conditions used, that both genes are being detected; however, this is not necessarily true because the DNA·DNA hybrids have a lower thermostability than the RNA·DNA hybrids (45).

Southern blot analysis at reduced stringencies (50°C in 5× SSPE) on *Saccharomyces cerevisiae* (Fig. 5) and *Drosophila melanogaster* (data not shown) genomic DNAs detect hybridizing bands in both of these species. At higher stringencies (65°C, 5× SSPE), crossreacting DNAs are detected by Southern blotting analysis in the genomic DNA of chicken and frog (data not shown). The detection of genomic insect and yeast DNA sequences homologous to cDNAs of the nuclear hnRNP C proteins strongly suggests that related proteins are present in these organisms.

DISCUSSION

The C proteins of the hnRNP complex are of considerable interest because they are essential for the packaging of hnRNA in the nucleus (4, 20, 21) and because they are the only proteins of the hnRNP particle so far shown to have a function in the splicing of pre-mRNA (49). We describe here the isolation of a cDNA clone for the human hnRNP C proteins and the preliminary characterization of the homologous mRNAs and genomic DNA sequences. This opens up the way for analyzing in detail the amino acid sequence, the mRNAs, and the structure, evolution, and expression of the genes for these major hnRNP proteins. By use of monoclonal antibodies, it has been shown that C proteins are present, and associated with hnRNA, in vertebrates from human to reptiles (26). Antibodies to the C proteins can be used to isolate the hnRNP complex from these organisms (21). The detection of hybridizing DNA sequences in insect and yeast cells strongly suggests that related proteins are also found in these organisms. It should therefore be possible to take advantage of the power of genetic tools that are available in these organisms to study the function of the C proteins and of the hnRNP complexes.

From the hybrid-selection and RNA-blot data, it appears that the C1 and C2 proteins are translated from two highly homologous mRNAs. The detected mRNAs are relatively small (about 1.4 kb and 1.9 kb) for the expected size of the proteins as estimated from their mobilities in $NaDodSO_4$/PAGE. It is possible that the mRNAs contain unusually short 5′ and 3′ noncoding sequences and a short 3′ poly(A) tail. It may also be that the proteins are actually smaller than their electrophoretic mobilities suggest and that particular features of the amino acid composition, sequence, or posttranslational modifications cause them to exhibit aberrantly low mobilities. The nucleotide sequence of full-length cDNAs for C1 and C2 is needed to resolve these questions.

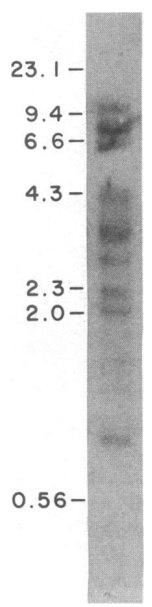

FIG. 5. DNA blot analysis of *S. cerevisiae* genomic DNA (10 μg) digested with *Eco*RI. Conditions were as described for Fig. 4, except that the hybridization temperature was 50°C in 5× SSPE.

The genomic blots of human DNA show multiple hybridizing restriction fragments with several enzymes. A general feature of mammalian multigene families is the presence of pseudogenes (46, 47), and it is possible that the genomic Southern blots of human and mouse are detecting such sequences in addition to functional C1 and C2 genes. Although less likely, it is also possible that other genes which code for related components of the hnRNP complex are being detected.

That partial genomic homologues of pHC4F4 exist in the lower eukaryote *S. cerevisiae* and in the invertebrate *D. melanogaster* suggests remarkable evolutionary conservation of hnRNP proteins. Indeed, Cruz-Alvarez *et al.* (48) have reported the isolation of a cDNA clone for HD40, an hnRNP protein from *Artemia salina*. This hnRNP cDNA also shows cross-hybridization to genomic DNA in divergent eukaryotes.

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