Volume 13 Number 11 1985 Nucleic Acids Research

Cloning of cDNA sequences for an *Artemia salina* hnRNP protein: evidence for conservation through evolution

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Received 18 March 1985; Revised and Accepted 14 May 1985

ABSTRACT

A cDNA clone was isolated for Artemia salina protein HD40, a component of heterogenous nuclear ribonucleoproteins. Enriched Artemia 15S poly(A)$^+$ RNA was used as a template and double-stranded cDNA sequences were inserted into the Pst I restriction endonuclease site of E. coli plasmid pBR322. Recombinant colonies were analyzed by positive hybrid selection of poly(A)$^+$ RNA that directs the synthesis of protein HD40 in an in vitro assay. In vitro translation of the mRNA selected by recombinant clone 87HD yields a protein that is immunoprecipitated by anti-HD40 antibodies and that comigrates with authentic HD40 on gel electrophoresis. Partial proteolysis of protein HD40 and the in vitro translated product selected by clone 87HD produces the same peptide patterns. The size of the cloned insert is about 820 bp. The length of HD40 mRNA as determined by Northern blot analysis, is about 1500 nucleotides. Southern blot analysis performed with DNA of different species (plant, avian, mammal) shows cross-hybridizing bands when probed with clone 87HD DNA suggesting that the HD40 gene is evolutionarily conserved.

INTRODUCTION

In eukaryotic cells, mRNAs and their nuclear precursors hnRNAs, are complexed with proteins giving rise to ribonucleoprotein particles (RNPs). The elucidation of the role of the proteins which bind RNA is essential for understanding the cellular processes involving hnRNA and mRNA (1). In electron micrographs of transcriptionally active chromatin, hnRNP can be seen as nucleoprotein fibrils with 20 nm beads spaced along their length (2-4). The individual hnRNP beads can be recovered from purified nuclei by extraction with isotonic buffers at pH 8.0-9.0 as monoparticles that sediment at 30-40S. The particles are about 20 nm in diameter, contain 8-10S fragments of rapidly labeled RNA and a number of proteins that comprise about 80-85°/o of the particle mass. A substantial fraction of the protein mass of hnRNP consists of a group of basic proteins (pI = 8.0- 9.0) with molecular weights between 30,000 and 45,000 characterized by similar

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amino acid compositions including a high content of glycine, the absence of cysteine, the presence of dimethylarginine, and a blocked NH_2-terminus (5-11). Proteins with these general properties are found in hnRNPs of many animal and plant species. They appear to be devoid of any enzymatic activity and are generally considered to function as structural components that condense and package hnRNA into the characteristic beads-on-a-string hnRNP structures. Nucleoprotein complexes that resemble native hnRNP can be reconstituted from exogenous RNA and this group of glycine-rich proteins (12,13). Mammalian hnRNPs contain only 4-6 polypeptides with different molecular weights as determined by SDS-polyacrylamide gel electrophoresis but some of these yield several subspecies upon isoelectric focusing. Peptide mapping experiments indicate that multiple species probably arise by post-translational modifications of a limited number of precursor polypeptides (11). Monoclonal antibodies against mouse hnRNP proteins detect not only most members of the group, but cross-react with corresponding proteins of several other animal species suggesting that the glycine-rich hnRNP proteins are related and evolutionarily conserved (14). A degree of conservation might be expected if they act as structural proteins in a way somewhat analogous to histones. The nature of the genes coding for hnRNP proteins, the pattern of their transcription, and the possibility that hnRNP proteins may be products of a limited number of genes, perhaps a conserved gene family, have not been analyzed.

An RNA binding protein that shares the properties of the glycine-rich hnRNP proteins of higher cells was purified to homogeneity from the primitive crustacean Artemia salina (15,16). This protein, termed HD40 (helix-destabilizing protein, Mr about 40,000), binds to, and progressively disrupts the residual secondary structure of single-stranded nucleic acids forming an extended nucleoprotein filament at a stoichiometry of one protein per 12-15 nucleotides. Upon addition of the protein in excess of this ratio, the filament becomes condensed into a beads-on-a-string structure similar in appearance to the beaded fibrils of hnRNP (16-18). Electrophoretic and immunological investigations demonstrate that protein HD40 is a major component of 30S hnRNP monoparticles isolated from the nuclei of A. salina (17). In this report we describe the isolation of a cDNA clone of HD40, analyze homologous sequences in A. salina DNA, and investigate the possibility of conservation of HD40 sequences throughout the phylogenetic scale.

EXPERIMENTAL PROCEDURES

Construction of cDNA Clones.

Extracts of developed A. salina cysts (San Francisco Bay brand) were prepared as described (15) except that all buffers were made 5 mM in vanadylribonucleoside complex to inhibit RNase activity. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose (Collaborative Research) chromatography (15); the yield was 0.68 mg from 25 g of dry cysts. The poly(A)$^+$ RNA was assayed for mRNA coding for HD40 by in vitro translation using a rabbit reticulocyte lysate system containing [^{35}S]-methionine (specific activity 1100 Ci/ mmole, New England Nuclear) and the translation products were immunoprecipitated with anti-HD40 (17) and staphylococcal protein A-Sepharose (Calbiochem) (19). Total translation products and the immunoprecipitated polypeptides were analyzed by polyacrylamide (10°/∘)-SDS gel electrophoresis (20), fluorographed, dried and autoradiographed. For the preparation of templates for the synthesis of cDNA, 80 μg of poly(A)$^+$ RNA was centrifuged through a linear sucrose gradient (10 to 30°/∘ in a buffer containing 10 mM Tris-HCl, pH 7.6, 1.0 mM EDTA and 10 mM methylmercuric hydroxide) for 15 h at 35,000 rpm at 4° (21) and each fraction was assayed for HD40 mRNA as above. The fraction containing HD40 mRNA (4.5 μg) was used for the synthesis of cDNA.

cDNA clones were constructed by insertion of ds cDNAs into the endonuclease Pst I site of plasmid pBR322. First-strand synthesis was according to the method of Kacian and Myers (22). The second strand was synthesized in a mixture (0.060 ml final volume) containing: 20 mM Tris-HCl pH 8.6, 60 mM KCl, 7 mM $MgCl_2$ 1 mM DTT, 0.2 mM of each dNTPs, ss cDNA, 8 μg/ml, and the Klenow fragment of DNA polymerase I (NEN), 7u/μg ss cDNA. The double-stranded cDNA was digested for 30 min at 37°C with nuclease S_1 (Boehringer) (10 units/μg of cDNA in 200 mM NaCl, 50 mM sodium acetate, pH 4.5, 1.0 mM $ZnSO_4$) and, after adjusting the pH to 7.0 and extraction, size-fractionated on a Sepharose 4-B column. A pool of fractions containing the ascending part of the excluded cDNA peak was poly dC-tailed by terminal transferase (New England Nuclear) (23) and annealed with an equimolar amount of poly dG-tailed endonuclease Pst I-cleaved plasmid pBR322 (24). Recombinant DNA was handled in accordance with the National Institutes of Health guidelines. E. coli RR1 cells were transformed as described (25) and recombinant colonies were identified by their resistance to tetracycline and sensitivity to ampicillin.

Screening of the cDNA library by translation of hybrid selected RNA was as described (26). Briefly, individual cDNA containing clones were grown to saturation and pools were made by inoculating 0.3 ml of each of seven cultures into 100 ml of NZCY medium (24) containing tetracycline (30 µg/ml). DNA was isolated by a clear lysate method (27), denatured, bound to nitrocelluose filters and hybridized to total Artemia poly(A)$^+$ RNA (80 µg/ml) as described (26). The hybridized RNA was eluted into Eppendorf tubes (two filters per tube) in the presence of 0.1 mg/ml of carrier yeast RNA, extracted with phenol-chloroform and precipitated with 2 vol of ethanol. Each pool, now representing RNA hybridized to 14 individual clones, was assayed by in vitro translation and immunoprecipitation by anti-HD40 antiserum as described above.

Peptide mapping by partial proteolytic digestion was performed as described by Melero et al. (28). After in vitro translation programmed by hybrid selected RNA followed by gel electrophoresis of the synthesized polypeptide, the protein band was excised, cut into small fragments and extracted overnight by shaking with 1.0 ml of 0.05 M NH_4HCO_3 and 0.5°/◦ SDS. The sample was centrifuged, the supernatant lyophilized, resuspended in 0.5 ml of H_2O, precipitated with 10°/◦ TCA and washed with acetone. Authentic protein HD40 (30 µg) was electrophoresed along with the product of in vitro translation and subjected to the same procedure. Digestions were carried out in 0.03 ml of a buffer containing 125 mM Tris-HCl, pH 6.8, 0.5°/◦ SDS and 10°/◦ glycerol (vol/vol) with S. aureus V8 protease (Miles), 0.5 µg/µg HD40 or with chymotrypsin (Sigma) 30 ng/µg HD40 at 25°C for 1 hour. After addition of SDS to 2°/◦, mercaptoethanol to 0.7M and bromophenol blue to 0.01°/◦, the samples were boiled for 2 min and electrophoresed in a 14°/◦ SDS polyacrylamide gel. The bands were visualized by Coomassie staining and autoradiography.

Northern and Southern Transfer and Hybridization.

A. salina poly(A)$^+$ RNA (2.5 µg) and poly (A$^-$) (25 µg) were denatured by heating at 65°C for 10 min in a buffer containing 20 mM 3-[N-morpholino]propanesulfonic acid, pH 7.0, 2.2M formaldehyde and 50°/◦ formamide, and subjected to electrophoresis in a 1°/◦ agarose gel containing 2.2M formaldehyde. Transfer to nitrocellulose filters, prehybridization and hybridization were performed as described (24,29). The cDNA insert from clone 87-HD was prepared by digestion of the cesium-chloride purified plasmid with endonuclease Pst I and electroelution from a 1°/◦ agarose gel. An internal 600 bp fragment was obtained by treating the insert with endo-

nucleases Bam HI and Nru I to remove the poly(dG·dC) tails. Restriction enzymes were from New England Biolab and digestions were carried out under conditions specified by the manufacturer. This fragment was ^{32}P-labeled by nick-translation (spec. act. 1 to 3 x 10^8 cpm per μg), and used as a probe.

For Southern hybridizations, the endonuclease Bam HI digestion products of DNA from A. salina, yeast, pea, chicken, mouse 3T3 cells, and human WI-18 cells, 10 μg each, were subjected to electrophoresis, transfer and hybridization as described (24). The probe was the same as prepared for the Northern hybridizations and filters were washed twice with 2 X SSC at room temperature and four times in 2 X SSC at 60° for "low stringency" conditions. For "high stringency" experiments filters were washed as described (24).

RESULTS AND DISCUSSION

Antibody to HD40 Detects a Single Polypeptide After in vitro Translation.

When total poly(A)$^+$ RNA from developed Artemia cysts (15) is translated in an in vitro rabbit reticulocyte system, about 0.1-0.2°/∘ of the synthesized protein is immunoprecipitated with anti-HD40 polyclonal monospecific antibodies (17), indicating that although HD40 is a major hnRNP protein, its mRNA is present in low abundance. As seen from Fig. 1, gel electrophoresis of the immunoprecipitate under denaturing conditions shows a single protein band of molecular weight 40,000 that migrates at the same rate as authentic HD40. Since the antibody clearly identifies the target protein, we decided to isolate HD40 cDNA clones by binding pools of recombinant plasmid DNA to nitrocellulose filters followed by hybridization to total poly(A)$^+$ RNA and the isolation of hybrid-selected RNAs; the RNAs are then assayed for their ability to program the synthesis of HD40 by in vitro translation and immunoprecipitation (26). This appears to be the most advantageous cloning procedure for a mRNA of a structural protein when no sensitive functional assay is available.

Molecular Cloning of the cDNA for HD40.

For the preparation of template for the synthesis of cDNA, total Artemia poly(A)$^+$ RNA was centrifuged through a linear sucrose gradient (see Experimental section). Most of the HD40 coding capacity sedimented at about 15S as estimated using rRNA as markers (not shown). Accounting for the systematic losses of the procedure, we estimate an enrichment of about 8-10 fold in this step. Double-stranded cDNA was synthesized using the

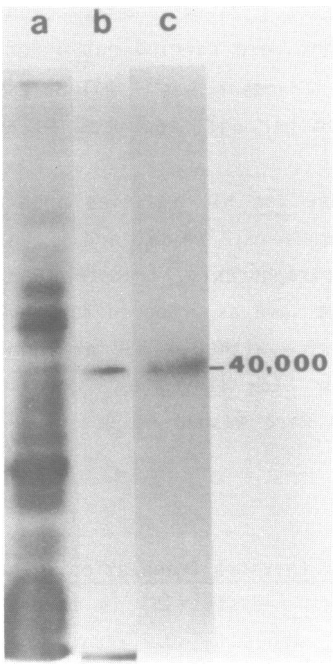

FIG. 1: SDS-polyacrylamide gel of [^{35}S]-methionine labeled products of in vitro translations programmed by total polyA$^+$ RNA before (lane a), and after (lane b), immunoprecipitation by anti-HD40; lane c, purified protein HD40 stained with Coomassie blue.

enriched template and inserted into the endonuclease Pst I site of plasmid pBR322 by the poly(dG) and poly(dC) tailing method as described (26,30). Transformation of E. coli RR1 cells with the recombinant plasmids yielded about 400 colonies that were resistant to tetracycline and sensitive to ampicillin and thus contained inserts. Assuming an 8-10 fold enrichment of the template, we estimated that about 1 clone in 250 would contain HD40 cDNA sequences. After the screening of 28 pools, each containing 14 individual clones (see Experimental section), one positive pool was identified by immunoprecipitation of the translation products (not shown). The positive pool was fractionated into its 14 components, each plasmid strain was grown separately and subjected to the mRNA selection and translation procedure (26). Figure 2 shows the results of polyacrylamide-SDS gel electrophoresis of the products coded for by mRNA selected by these clones before and after immunoprecipitation with anti-HD40 antibodies. Only one clone, termed 87HD, produced a protein band of appropriate mobility (Fig. 2A, lane h) and, furthermore, this was the only product immunoprecipitated by anti-HD40 (Fig. 2B). The absence of detectable protein bands for clones other than 87HD after a relatively short exposure (Fig. 2A) may be due to the presence of

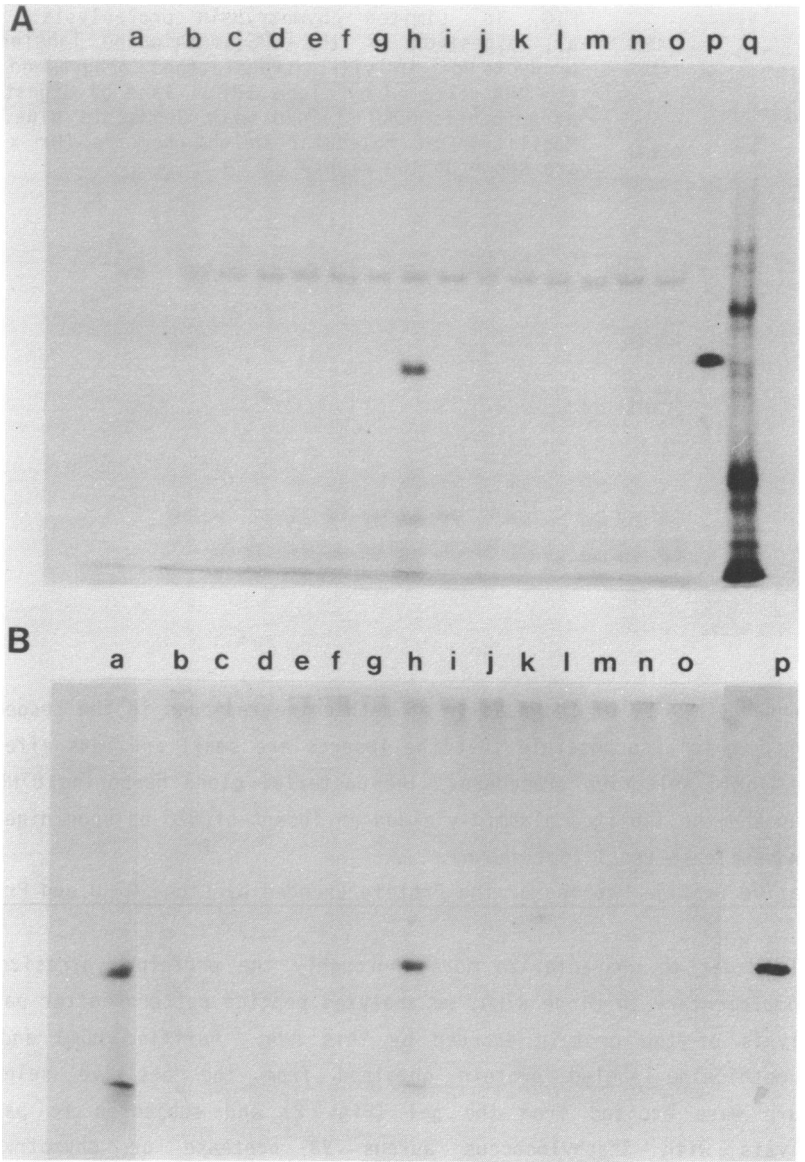

FIG. 2: SDS-polyacrylamide gel of [^{35}S]-methionine labeled products of *in vitro* translations programmed by 14 hybrid selected RNAs from the positive clone pool (see text for explanation). The translation products were electrophoresed before (panel A) and after (panel B) immunoprecipitation by anti-HD40. In both panels, lanes b to o represent translation of RNAs selected by the 14 individual clones of the pool and lane p contains protein HD40 marker; lane a in panel A contains *in vitro* translation products in the absence of exogenous RNA; lane q in panel A and lane a in panel B represent translation of total poly(A)$^+$ RNA.

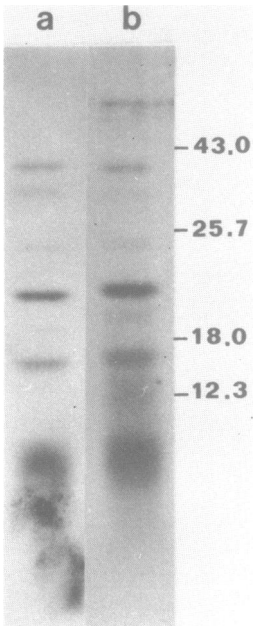

FIG. 3: Limited chymotrypsin proteolysis. Lane a), digestion of the [^{35}S]-methionine labeled products of in vitro translations programmed by the RNA selected by clone 87HD; lane b) digestion of authentic HD40 stained with Coomassie blue. Mobilities of molecular weight markers (Mr x 10^{-3}) are shown on the right.

low abundance mRNAs, or the paucity of methionine residues in the respective proteins; it is also possible that the inserts are small and less effective in the hybrid selection procedure. The bacterial clone harboring 87HD was grown up and the isolated plasmid yielded an insert of 820 bp upon digestion with endonuclease Pst I (not shown).

Comparative Peptide Mapping of the Protein Encoded by Clone 87HD and Protein HD40.

In order to characterize more thoroughly the protein synthesized by mRNA complementary to clone 87HD, we analyzed peptide patterns after partial proteolysis of the protein encoded by this RNA. Purified HD40 and the [^{35}S] methionine-labeled protein obtained from the positive selection procedure were excised from the gel (Fig. 2) and subjected to partial proteolysis with Staphylococcus aureus V8 protease or chymotrypsin. Digestion products were electrophoresed in a polyacrylamide-SDS gel and the gel was stained prior to autoradiography. Methionine labeled bands were compared with those generated by the partial proteolysis of the authentic unlabeled protein. As seen in Fig. 3, the labeled and stained patterns after chymotrypsin digestion are virtually identical. The same result was obtained with S. aureus V8 protease (not shown).

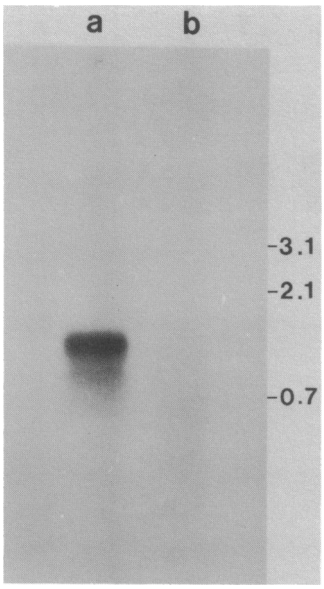

FIG. 4: Northern blot analysis of A. salina polyA$^+$ RNA (lane a) and polyA$^-$ RNA (lane b), probed with 87HD DNA.

Taken together, these experiments demonstrate that clone 87HD contains sequences coding for protein HD40 since i) it selects by hybridization for an mRNA that programs the synthesis of a protein with the same molecular weight as HD40, ii) the in vitro synthesized protein is the only product immunoprecipitated with anti-HD40 antibodies, and iii) partial peptide maps of this protein correspond to those produced by authentic HD40. This is, to our knowledge, the first cDNA clone of an hnRNP protein.

Estimation of the Size of HD-40 mRNA.

Samples of poly(A)$^+$ and poly(A)$^-$ RNA from developing A. salina were fractionated according to size by gel electrophoresis, transferred to a nitrocellulose filter and hybridized to the ^{32}P labeled insert of plasmid 87HD (see Experimental section). The probe detects a single band that is approximately 1500 nucleotides in length (Fig. 4). The size of the HD40 mRNA estimated by the Northern blot is consistent with its sedimentation at about 15S in the methylmercury/sucrose density gradient used to enrich the RNA prior to cloning. Since the Mr of HD40 corresponds to a protein coding sequence of about 1200 nucleotides, it appears that about 20°/∘ of the mRNA represents non-translated sequences.

There is virtually no hybridization with poly(A)$^-$ RNA consistent with the fact that the probe was cloned from poly(A)$^+$ RNA, and confirming

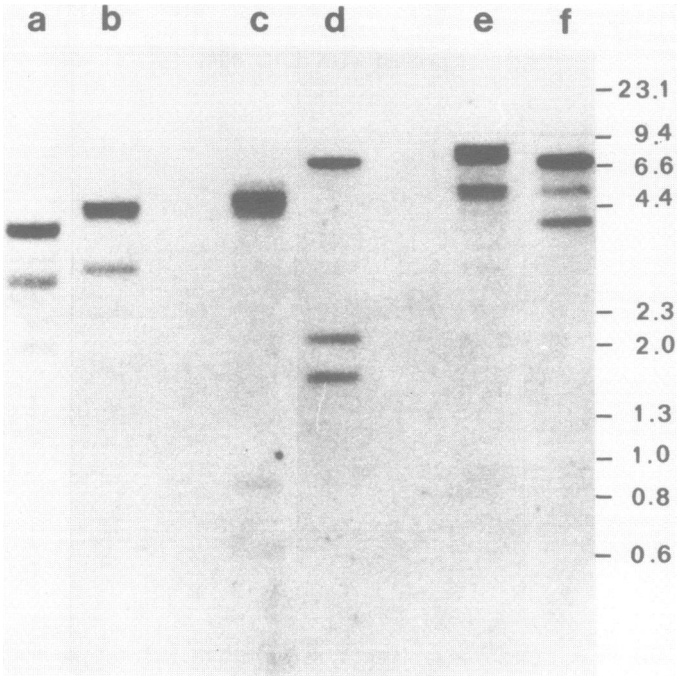

FIG. 5: Southern blot analysis of A. salina DNA isolated from San Francisco Bay brand cysts (lanes a, c and e) and from Great Salt Lake brand cysts (lanes b, d and f). The DNAs were digested with endonucleases Eco RI (lanes a and b); Hind III (lanes c and d) and Bam HI (lanes e and f). Hybridization was to probe 87HD DNA. Mobilities of size markers (in Kb) are shown on the right.

the presence of polyA in the mRNA for HD40. The presence of poly(A)$^-$ mRNA in Artemia has been reported (31).

Pattern of HD40 Homologous Nucleotide Sequences in A. salina DNA.

A. salina DNA (San Francisco Bay brand) was digested with restriction enzymes Eco RI, Hind III and Bam HI, electrophoresed and analyzed by Southern blotting using 87HD DNA as probe. As shown in Fig. 5, in lanes a, c and e, several bands of different intensities can be observed with each enzyme. Since the cDNA probe contains one cleavage site for Eco RI and no cleavage sites for the two other restriction enzymes (Hind III and Bam HI, unpublished observations) there are at least three alternative ways to explain this result: i) the cleavage sites could occur in introns, ii) there could be a family of related genes, and iii) DNA restriction site polymorphisms, since the cysts come from a wild, unbred population.

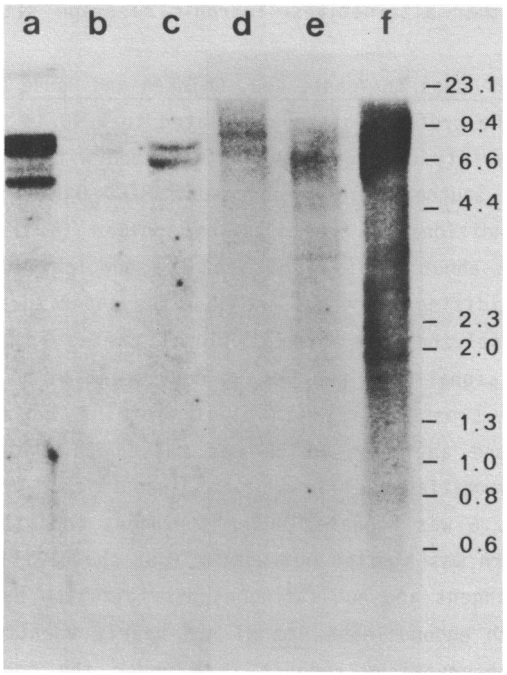

FIG. 6: Southern blot analysis of DNAs isolated from a, A. salina, San Francisco Bay brand; b) yeast; c) pea; d) chicken; e) mouse 3T3, and f) human WI-18 cells. The DNAs were digested with endonuclease Bam HI. Hybridization was to probe 87HD DNA under less than stringent conditions (see Experimental section). Mobilities of size markers (in Kb) are shown on the right.

To explore the third alternative, we have analyzed the patterns of digestion of DNA from Great Salt Lake Artemia salina, another wild, unbred population of the same species (Fig. 5, lanes b, d and f). The two geographically isolated bisexual populations are cross-fertile but have different biomasses and also differ with respect to a number of biochemical characteristics (32,33). It should be pointed out that all the experiments reported here as well as those on the isolation and properties of protein HD40 (15-18), were carried out using San Francisco Bay brand Artemia. Artemia cells grown in cultures are not available. As seen from Fig. 5, the patterns of digestion of DNA from the two populations do not coincide, suggesting the existence of strain-specific and/or intra-strain restriction site polymorphisms. Cloning of Artemia genomic sequences and analysis of their restriction maps and hybridization properties will most likely show

whether the two other alternatives--intronic cleavage sites and/or a gene family--also exist.

Analysis of Related Genes in Yeast, Pea, Chicken and Mouse DNA.

To explore the presence of genes related to HD40 in different species, DNA from a wide variety of organisms was digested with endonuclease Bam HI and analyzed in a Southern blot probed with 87HD DNA (Fig. 6). The less than stringent conditions of hybridization applied in this experiment are expected to detect about 60-70°/∘ of sequence homology (24). As shown in Fig. 6, cross-hybridizing DNA bands are observed under these conditions with all the species analyzed, the weakest signal coming from yeast DNA. The relatively strong signal with pea DNA is consistent with the fact that the anti-HD40 antibody cross-reacts with plant proteins (wheat and onion); on the other hand, the antibody, which was raised in the rabbit, does not cross-react with mammalian nuclear proteins (unpublished observations). The experiment of Fig. 6 was repeated under stringent conditions of hybridization and the pattern was similar but weaker (not shown). It is essential to note that the stringent and non-stringent hybridization patterns of Artemia DNA restricted with endonuclease Bam HI are nearly identical (cf. Figs. 5e and 6a). These observations suggest that under the conditions employed, specific HD40-related sequences are being detected in the genomes of the organisms analyzed. Southern analysis of human DNA (Fig. 6f) is very different from that of all the other organisms examined since it shows, in addition to a high background, about 8-10 defined fragments ranging in size from 0.8Kb to about 15Kb, each giving a strong signal. This experiment was repeated several times with different preparations of human DNA, previously assayed for intactness, and each time the same pattern was observed. Essentially the same pattern was also seen under stringent conditions of hybridization except that the signals were weaker. The simplest explanation of this result would be the presence in human DNA of a moderately repetitive sequence homologous to the Artemia probe. The presence of a large number of pseudogenes is a possibility since this phenomenon has been recently observed with respect to an enzyme, glyceraldehyde 3-phosphate dehydrogenase, coded by a single functional gene (34). The existence of avian and mammalian genes related to HD40 opens up the possibility of analyzing the organization and expression of hnRNP protein genes of higher organisms.

The primary structure of protein HD40 and of other hnRNP proteins is not known and even a comparison of their N-terminal sequences is hampered since the N-termini of all major hnRNP proteins are blocked (11,14,15). We

expect that the amino acid sequence of protein HD40 deduced from sequencing of cDNA clones will contribute to an understanding of how the different functional domains of the protein contribute to RNA-protein and protein-protein interactions that result in the formation of hnRNP particles (18).

ACKNOWLEDGEMENTS

We wish to thank Drs. A. Villasante and J.O. Thomas for helpful discussions, Dr. Razziudin for assistance with the chymotrypsin digestion, and Dr. M. Rush for critical reading of the manuscript. This work has been supported by grants CA16239 to A.P. and GM23705 to W.S. A.P. is an Irma Hirschl/Monique Weil Caulier award recipient.

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