Polypeptide components of Drosophila small nuclear ribonucleoprotein particles

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ABSTRACT

In eukaryotes splicing of pre-mRNAs is mediated by the spliceosome, a dynamic complex of small nuclear ribonucleoprotein particles (snRNPs) that associate transiently during spliceosome assembly and the splicing reaction. We have purified snRNPs from nuclear extracts of Drosophila cells by affinity chromatography with an antibody specific for the trimethylguanosine (m3G) cap structure of snRNAs U1-U5. The polypeptide components of Drosophila snRNPs have been characterized and shown to consist of a number of proteins shared by all the snRNPs, and some proteins which appear to be specific to individual snRNP particles. On the basis of their apparent molecular weight and antigenicity many of these common and particle specific Drosophila snRNP proteins are remarkably conserved between Drosophila and human spliceosomes. By probing western blots of the Drosophila snRNP polypeptides with a number of antisera raised against human snRNP proteins, Drosophila polypeptides equivalent to many of the HeLa snRNP-common proteins have been identified, as well as candidates for a number of U1, U2 and U5-specific proteins.

INTRODUCTION

In eukaryotes the excision of intervening sequences from pre-mRNA is catalyzed in a large ribonucleoprotein complex, the spliceosome. The spliceosome is composed of at least 30 distinct proteins and the five major small nuclear RNA (snRNA) molecules U1, U2, U4, U5 and U6. The U snRNAs can be recovered from nuclear extracts as four individual small nuclear ribonucleoprotein (snRNP) complexes, the monomeric U1, U2 and U5 snRNPs and the dimeric U4/U6 snRNP in which the U4 and U6 snRNAs are found base paired (1,2).

The protein composition of the snRNP complexes has been studied in detail in human and rodent nuclear extracts (3,4). Eight distinct polypeptides, B, B', D1, D2, D3, E, F and G, with apparent molecular weights of between 9 and 29 (M,×10^3), are common to all four snRNPs and form a common snRNP core. In addition, individual snRNPs have been shown to contain characteristic particle-specific proteins, denoted 70K, A and C for U1; A' and B" for U2; and 15, 40, 52, 100, 102, 116 and 200K for U5. The U5 snRNP has a particularly complex protein composition and sediments through glycerol gradients more rapidly than the other snRNPs (5). An even larger tripartite U4/U5/U6 complex can be isolated under low salt conditions and contains at least four further polypeptides (4,6-8).

Antisera from patients with a variety of connective tissue diseases recognize many of the snRNP polypeptides. Anti-RNP autoantibodies, from patients with mixed connective tissue disease (MCTD), react with the U1-specific proteins A, C, and 70K. Anti-Sm sera from patients with systemic lupus erythematosus (SLE) immunoreact with the core (Sm) proteins B', B and D (9,10), with variable reactivity to D1, D2 and D3 (11), and less frequently with E, F and G (12). These sera have been used to study the composition of snRNPs from a variety of species by immunoprecipitation and immunoblotting. These studies indicate that the protein composition of snRNPs is remarkably conserved throughout eukaryotes (reviewed 3) as indeed are the sequences of the U snRNAs (reviewed 13).

Purification of snRNPs has been facilitated by the use of the human autoantibodies, but more successfully by the use of monoclonal antibodies which recognize modified nucleosides peculiar to the snRNAs. In particular antibodies which recognize the highly methylated nucleoside 2,2,7-trimethylguanosine (m3G), which is found as a 5'-cap on U1, U2, U4 and U5 snRNAs, allow a single step purification of m3G-capped snRNPs from nuclear extracts by immunoaffinity chromatography. Native snRNPs can be recovered by desorption with an excess of m3G (or the cross-reactive nucleoside 7-methylguanosine, m7G) or by elution with salt (14-17).

The Drosophila U snRNAs closely resemble those of vertebrates in size, structure and nucleotide sequence (16, reviewed 13). Anti-Sm and anti-RNP antisera have identified a number of low molecular weight potential snRNP polypeptides (19,20). In this study we have used a monoclonal antibody which recognizes the 5'-nucleotide cap of snRNAs (16) to immunoaffinity purify snRNPs from nuclear extracts of Drosophila cells in order to identify their protein components and to compare them to those of HeLa cells.
MATERIALS AND METHODS

Cell growth and preparation of nuclear extracts

The Drosophila melanogaster Schneider 2 cell line was propagated at 22°C in suspension in Schneider's (modified) Drosophila Medium (purchased from Imperial Laboratories, UK) supplemented with 5% foetal calf serum, 100U/ml penicillin and 100μg/ml streptomycin. Nuclear extracts were prepared by the method of Dignam et al., (21) without homogenization of cells and with the addition of 0.5% v/v NP40 to disrupt the cytoplasmic membrane. HeLa cell nuclear extracts and purified snRNPs were kindly provided by Silke Börner.

Anti-m3G immunoaffinity chromatography of snRNPs

Affinity purification of m3G-capped snRNPs from nuclear extracts was performed by using mAbo H-20 bound covalently to CNBr-activated Sepharose 4B essentially as described (16). Nuclear extract prepared as above was passed over a 5ml H-20-Sepharose column and bound RNA-protein complexes were desorbed with 20mM m7G.

Fig. 1. Immunoaffinity purification of Drosophila snRNPs. Analysis of fractions upon chromatography over H-20-Sepharose. (A) Coomassie stained proteins resolved on a 15% polyacrylamide/SDS gel compared with HeLa snRNP proteins (H) and size markers (S) (M, ×10^-3). L, nuclear extract loaded onto column; F, unbound flow through; W, initial wash after loading; E, eluate desorbed on addition of m7G; U, proteins in 6M urea wash to regenerate column. (B) Ethidium bromide stained RNA resolved on a 6% polyacrylamide/urea gel. Fractions as above except that W, wash fraction was sampled after extensive washing of the column prior to desorption. The identities of U1, 4, 5 and 6 RNAs were determined by hybridizing a northern blot of the gel with unique oligonucleotide probes. Positions of SS rRNA and 4S rRNA are indicated.

Anti-sera

Rabbit: anti-FP8.1 raised against a portion of yeast U5 snRNP specific protein PRP8 fused to β-galactosidase and affinity-purified against the same portion of PRP8 fused to the trpE protein (22).

Monoclonal antibodies: Y12 anti-Sm type (9); 7.13 anti-HeLa D1 (23); H-20 anti-m3G (16); 4G3 anti-HeLa U2B' (24); H111 anti-HeLa U1 70K, H304 anti-HeLa U1 and H386 anti-HeLa U1 70K and U5 100K (8,25).

Patient 7, E74 and Plasma D anti-Sm sera from patients with systemic lupus erythematosus were kindly provided by Prof. H.Peter, Freiburg.

Alkaline phosphatase-conjugated secondary antibodies (against rabbit, human or mouse IgG or mouse IgM antibodies) were purchased from Promega and Bio-Rad.

RNA and protein analysis

Samples were extracted with 1 volume of PCA (phenol, chloroform, isoamylalcohol 50:50:1) with 0.5% (w/v) SDS. Following phase separation by centrifugation RNAs were recovered from the aqueous phase by ethanol precipitation. Proteins were precipitated from the organic phase with 5 volumes of acetone.

RNAs were fractionated by electrophoresis through 10% polyacrylamide gels containing 7M urea, 10mM Tris-Borate pH8.2 and 2mM EDTA (TBE). RNA was visualized by staining with ethidium bromide (0.5μg/ml) or with silver (26). RNA was transferred to nylon membrane (Amersham) by electrophoresis in 0.5×TBE for 3h at 45V and fixed by UV irradiation. U snRNA-specific oligonucleotides were 5'-end labelled using polynucleotide kinase and hybridized to pre-blocked filters in 6×SSC (3M NaCl, 3M trisodium citrate), 5×Denhardt’s solution, 100μg/ml denatured salmon sperm DNA for 2h at 4°C and washed briefly in 6×SSC at 20°C before autoradiography. Oligonucleotides were synthesized by OSEWEL DNA Service, Edinburgh University, and were directed against: Drosophila U5

Fig. 2. Comparison of Drosophila (D) with HeLa (H) snRNP proteins resolved on a 12% acrylamide/SDS gel polymerized in the presence of high TEMED concentration (see Methods). The migration of size standards is shown (14 – 205 M, ×10^-3) and the major HeLa snRNP proteins are indicated.
bases 68–83 GACTCTTAGAGTGTC; Human U4 bases 58–75 GGAAAAGTTTCTAATTAG; Human U6 bases 78–95 GGGTCAGAACAGTTTGC.

Proteins were fractionated by SDS-gel electrophoresis (27). In order to resolve small proteins, in particular the HeLa D proteins, gels were polymerized where noted in the presence of high TEMED concentrations (0.04% compared to 0.01%, ref. 11). Protein bands were detected by staining with Coomassie brilliant blue G250 or were transferred to nitrocellulose by electrophoresis overnight at 25V in 25mM Tris, 19mM glycin (28). Blots were then probed with sera or monoclonal antibodies as described previously (25) and antibodies detected with alkaline-phosphatase-conjugated secondary antibodies and chromogenic substrates (Promega).

Glycerol gradient centrifugation
Affinity purified snRNP fraction (200μg protein, prepared as described above) was layered onto a linear 10–30% (v/v) glycerol gradient in a buffer containing 20mM Hepes-KOH pH8, 150mM KCl, 1.5mM MgCl2, 0.5mM PMSF, 0.5mM DTT, and 2μg of leupeptin per ml. The gradient was centrifuged in a Beckman SW40 rotor at 29 krpm for 18h. 500μl fractions were collected and PCA extracted for analysis as described above.

RESULTS

Purification of Drosophila snRNPs from nuclear extracts by chromatography on H-20 affinity columns

Nuclear extract prepared from 0.5 × 10^10 Drosophila cells was purified by H-20 immunoaffinity chromatography (Materials and Methods) and yielded 620μg of protein upon desorption of bound snRNPs (see Figure 1A). The yield was somewhat lower than routinely achieved with HeLa cell extracts. Analysis of the RNA eluted from the H-20 column revealed a marked enrichment of the U snRNAs from the nuclear extracts (Figure 1B).

Characterization of Drosophila snRNP proteins in relationship to their human counterparts

The protein component of purified Drosophila snRNPs was analysed by SDS-gel electrophoresis and compared with purified snRNPs from HeLa cells. A larger number of individual proteins were enriched from Drosophila in comparison with HeLa cells from which around 30 major proteins were selectively enriched (Figures 1A, 2). As a consequence, few direct correlations between the sizes of Drosophila and human proteins are immediately apparent. Drosophila and HeLa snRNP proteins were transferred to nitrocellulose after gel electrophoresis and probed with a variety of antisera reactive to HeLa snRNP proteins in order to investigate the relationship between the affinity purified Drosophila and human proteins. When strips were probed with anti-Sm sera from three human SLE patients a number of Drosophila Sm proteins were identified (Figure 3A). On the basis of their apparent molecular weight these may include at least two D proteins, D^U and D^L (D^L may in fact be a doublet, see lane 4 and also Figure 2A), at least one potential B protein, a potential A protein, several proteins with similar mobility to the HeLa U1 70K protein and a doublet at about 100K (lane 4). The exact relationship of D^U and D^L to the HeLa D1, 2 and 3 proteins is not clear but E 74 serum, which recognizes human D1 and D3, only recognizes the more slowly migrating Drosophila protein D^U (lane 1) whilst sera recognizing all three HeLa D proteins also recognize D^L (lanes 2 and 4).

Various monoclonal antibodies and rabbit antisera which react with specific snRNP proteins were also used as probes...
(Figure 3B). These antibodies confirmed the existence of at least two distinct D proteins $D^U$ and $D^L$ (lanes 1 and 2). Again the relationship of $D^U$ and $D^L$ to D1, D2 and D3 could not be unambiguously assigned since in this case antibodies raised against D1 (7.13) recognized $D^L$ (lane 2). The mobility of the D proteins was clearly affected by the composition of the acrylamide gel used (Materials and Methods, 11). In a 12% acrylamide gel polymerized with high concentrations of TEMED, $D^U$ and $D^L$ ran with similar mobility to HeLa D proteins (Figure 3A lanes 1, 2; Figure 3B lanes 1, 2), but in a 14% acrylamide, low TEMED gel the Drosophila D proteins migrated more slowly than the HeLa counterparts (Figure 3A lanes 3, 4).

As was observed with the Human Sm antisera only a single Drosophila B protein was detected by the anti-Sm monoclonal antibody Y12 (lane 1). The antibodies H111 and H386 (lanes 3, 4) define a unique Drosophila band related to the HeLa U1 70K protein whereas the human anti-Sm sera (Figure 3A) indicated that there may be multiple post-translationally modified variants of this protein as has been observed in HeLa cells. Antiserum raised against a portion of the yeast U5 snRNP specific protein PRP8 (22) recognized large 200–260kDa proteins in both HeLa and Drosophila snRNPs (lane 6) as previously reported (29–31). Antiserum raised against other regions of the yeast protein (22) also recognized both the HeLa and Drosophila protein (30, results not shown). A monoclonal (4G3) raised against the HeLa U2-specific B' protein (24) recognized a similarly sized Drosophila protein (lane 7).

A monoclonal antibody specific to the HeLa A protein (H304, lane 5) and rabbit antipeptide antisera to the HeLa C and G proteins (results not shown) failed to recognize equivalent Drosophila proteins, perhaps reflecting lack of conservation of these epitopes between Drosophila and humans. Results from these and the later analyses are summarized in Table 1.

### Resolution of the Drosophila U5 snRNP particle by sedimentation through glycerol gradients

The immunoaffinity purified Drosophila snRNP fraction was subjected to sedimentation analysis in a linear 10–30% (v/v) glycerol gradient in order to investigate whether rapidly and slowly sedimenting particles could be resolved as has been demonstrated for HeLa snRNPs particles (5). When the protein content of the glycerol gradient fractions was analysed by gel electrophoresis a pattern remarkably similar to that observed with HeLa extracts was found (Figure 4A). The snRNP particles appear to have sedimented in two different size ranges; the rapidly sedimenting particles (fractions 14–17) appear to correspond to the 20S U5 snRNPs of HeLa (5) whilst the more slowly sedimenting particles (fractions 4–10) resemble in composition

![Figure 4](image-url)
Drosophila prominent polypeptide B' common weight 240-260K HeLa with these proteins raised are at correspond in degradation HeLa. Alternatively monoclonal antibodies to B (the faster migrating species, B'), corresponds to the B protein recognized by anti-Sm sera; Figure 3A lanes 1.2 and 4); the two D proteins D^U and D^L identified immunologically above, and two proteins with similar sizes to the HeLa core proteins E and F. Whilst a protein of similar size to HeLa G is present in the total Drosophila snRNP fraction (arrowed in lane L), this band is not discernible in either of the glycerol gradient fractions.

The more slowly migrating complexes (fractions 4-10) appear to contain the U1 snRNP specific proteins A and 70K. Again a number of proteins with apparent size of about 70 000 can be distinguished, perhaps indicating that multiple post-translationally modified variants of this protein are found in Drosophila as in HeLa. Alternatively this protein could have suffered proteolytic degradation during purification. Of the three candidate proteins in Drosophila with M, 54 000, 56 000 and 61 000 it appears to be the 56 000 species which is recognized by the anti-70K monoclonal antibodies. Whilst there is no protein of similar molecular weight to the HeLa C protein (M, 22 000) a prominent polypeptide of M, 14 500 may be the equivalent Drosophila protein. The Drosophila equivalent of the U2-specific protein B' (M, 28 000) was shown to be present in this fraction by western blotting and probing with the antibody 4G3 (result not shown). The Drosophila B' protein identified by western blot comigrates with the larger of the two candidate B proteins, B^U, which is present in both fractions of snRNPs in Figure 4C.

The rapidly sedimenting particles in fractions 14-17 resemble HeLa U5 snRNPs in protein composition. The high molecular weight 240-260K protein which is recognized by antibodies raised against the yeast U5-specific protein PRP8 is present, as are at least two proteins of M, 105-120 000 which might correspond to the 100, 102 and 116K proteins in HeLa. Two proteins of similar size to the HeLa 40K and 52K proteins also co-sediment with these Drosophila U5 snRNP particles.

Probing western blots of these glycerol gradient fractions with the human anti-Sm antisera confirmed the presence of the common proteins B^L, D^U and D^C in both fractions 4-10 and 14-17, whilst the Drosophila equivalents of the U1-specific 70K and A, and U2-specific B' proteins where restricted to fractions 4-10, and the U5-specific 100 and 200K proteins to fractions 14-17 (results not shown).

**DISCUSSION**

*D. melanogaster* provides an ideal model higher eukaryote in which to study the mechanism and regulation of pre-mRNA splicing. An increasing number of genes have been described where splicing of their transcripts is regulated in a developmental, sex or tissue-specific manner (see references 32-37 for examples) and a number of genes that regulate splicing have been identified, several of which encode proteins with RNA binding motifs resembling those found in mammalian snRNP proteins (38-41). Furthermore, nuclear extracts prepared from embryos or cultured Drosophila cells have been shown to accurately splice mRNA precursors by a similar mechanism to that observed in yeast and mammals (42).

In order to characterize the Drosophila splicing machinery we have purified snRNP particles from cultured Drosophila cell extracts by immunoaffinity chromatography and analysed their protein composition. The polypeptide components of Drosophila snRNPs appear remarkably similar to those characterized in HeLa cells. On the basis of molecular weight, antigenicity and particle density-sedimentation we have identified Drosophila proteins corresponding to most of the characterized HeLa polypeptides (summarized in Table 1).

Eight proteins have been identified in HeLa extracts which are common to all snRNP particles: B', B', D1, D2, D3, E, F and G. On the basis of molecular weight Drosophila has proteins corresponding to most if not all of these, and at least three proteins B', D^U and D^D are immunologically related to the equivalent HeLa proteins. Indeed the Drosophila B and D proteins identified in this study probably correspond to proteins previously identified by cross-reactivity with human anti-Sm and anti-snRNP antisera with reported molecular weights of 18 and 26 000 (19) and 14 and 26 000 (20).

Proteins which are specific to U1, U2 and U5 snRNPs are also conserved between humans and Drosophila. A counterpart to the HeLa U5-specific 200K protein which cross reacts with antibodies raised against the yeast PRP8 protein (29-31) is found in Drosophila U5-containing particles, as are at least two proteins of about M, 100-120 000 and other proteins potentially corresponding to the less well characterized 40 and 52K HeLa U5 proteins.

Drosophila proteins corresponding to the HeLa U1 snRNP specific 70K and A proteins were recognizable on the basis of their size and antigenicity, but no protein of equivalent molecular weight to the HeLa C protein could be detected. An abundant and potentially U1-specific protein of M, 14 500 was, however, resolved by density centrifugation and may be the Drosophila counterpart to C (Figures 4A,C).

A protein of similar size to the HeLa U2-specific B' protein was recognized by an antibody raised against the human protein (24). This antibody also recognizes a protein of M, 27 000 present in plant nuclear extracts and the gene encoding B' has recently been cloned from potato and shown to be highly conserved between the animal and plant kingdoms (43). A candidate gene coding for either the U2-specific A' or B' proteins has recently been cloned from Drosophila (44).

As in HeLa extracts, a number of electrophoretically distinct species of around 70 000 were detected. The most abundant of these had an apparent molecular weight similar to that of the most abundant HeLa 70K protein (61-62 000 in this system, Figures 2: 4A). However, the species recognized by monoclonal antibodies raised against the HeLa 70K protein had an apparent molecular weight of 56-58 000 (Figure 3B). A further species of 52-54 000 was also detected (Figure 2) and all three or more bands, perhaps representing modified or partially degraded versions of 70K, were recognized by some anti-Sm sera (Figure 3A). The gene encoding the Drosophila 70K protein has been cloned and found to encode a protein of predicted M,
53 000, the amino-terminal region of which is 68% identical to the human and Xenopus 70K proteins (45). Whilst there is no evidence in Drosophila that alternative RNA processing pathways give rise to 70K variants it is possible that some different forms of 70K may be generated by variable post translational modifications as has been reported for the mouse and human 70K proteins (46).

Until now the biochemical purification of snRNP particles for their characterization has largely been restricted to rodent and human cell types. Studies on a disparate range of species, primarily using autoimmune antibodies, have identified proteins corresponding to D, E, F and G in most systems (reviewed 3). This study shows that in Drosophila the conservation of the proteins involved in the mechanism of splicing extends much further. The striking degree of similarity between the snRNP proteins found in Drosophila and humans is perhaps not unexpected, given the evolutionary constraints on maintaining an accurate and efficient splicing apparatus.

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