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A yeast cap binding protein complex (yCBC) acts at an early step in pre-mRNA splicing

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ABSTRACT

The function in splicing of a heterodimeric nuclear cap binding complex (yCBC) from the yeast *Saccharomyces cerevisiae* has been examined. Immunodepletion of splicing extracts with antibodies directed against one component of the complex, yCBP80, results in the efficient co-depletion of the second component, yCBP20, producing CBC-deficient splicing extract. This extract exhibits strongly reduced splicing efficiency and similar reductions in the assembly of both spliceosomes and of the earliest defined precursors to spliceosomes, commitment complexes. The addition of highly purified yCBC substantially restores these defects. These results, together with other data, suggest that CBCs play a highly conserved role in the recognition of pre-mRNA substrates at an early step in the splicing process.

INTRODUCTION

The removal of intervening sequences (introns) from pre-mRNAs is an essential step in the expression pathway of many genes. Introns are recognized by a subset of the splicing factors and assembled into a large multi-component ribonucleoprotein complex called the spliceosome, where they are accurately removed from the pre-mRNA. The spliceosome consists of U1, U2, U4/U6 and U5 small nuclear ribonucleoproteins (snRNPs), together with numerous non-snRNP splicing factors. Within the spliceosome two *trans*-esterification reactions occur which result in production of the mature mRNA and intron lariat. Efficient splicing of an intron depends on recognition of the *cis*-acting nucleotide sequences present in the pre-mRNA at the 5′-splice site, branch point and 3′-splice site (1–3).

Both *in vivo* studies in yeast and the development of extracts capable of *in vitro* splicing from both yeast and higher eukaryotes has allowed several steps of spliceosome assembly to be characterized and some of the components required for these steps to be defined. In yeast the first complex to be detected is the ‘commitment complex’, which requires the 5′-splice site and branch point sequences and forms in the absence of ATP (4–6). This complex is so named as its formation commits the pre-mRNA to the subsequent spliceosome assembly and splicing pathway. Two components present in this complex and required for its formation are the U1 snRNP, which base pairs with the 5′-splice site (6–8), and MUD2, which is thought to be the yeast homologue of the mammalian splicing factor U2AF (U2 snRNP auxiliary factor) (9). A complex with similar characteristics and components to the yeast commitment complex has also been detected and characterized in mammalian extracts, the early or E complex (10,11). The commitment or E complexes are the substrate for the addition of the U2 snRNP, a step which requires the hydrolysis of ATP. The U4/U6.U5 tri-snRNP then joins this complex to form the mature spliceosome in which the chemical reactions of splicing occur (1–3,12).

Much has been learned of the contributions of the spliceosomal U snRNAs to spliceosome assembly, RNA recognition and cleavage of the splice sites, however, the role of non-snRNP splicing factors in recognition of the pre-mRNA is less well characterized. The cap structure, which is characteristic of RNA polymerase II transcripts, has been shown to play an important role in pre-mRNA splicing in higher eukaryotes (13–17). This activity of the cap is mediated by a nuclear cap binding protein complex (CBC). CBC has been previously characterized and cloned. It consists of two subunits, CBP80 and CBP20, both of which are required for cap binding (18–20). CBC is required for nuclear export of U snRNAs from the nucleus of *Xenopus* oocytes (19) and efficient pre-mRNA splicing in HeLa cell nuclear extracts, where it functions early in spliceosome assembly (18). A more detailed analysis of the splicing defect has shown that CBC is required for efficient association of U1 snRNP with the 5′-splice site of the pre-mRNA in what may be one of the earliest steps in pre-mRNA recognition (21).

The homologues of CBP80 and CBP20 have been identified in the yeast *Saccharomyces cerevisiae*. For reasons of clarity the yeast homologues of CBP80 and CBP20 or yeast CBC will be prefixed by the letter y. yCBP80 was found as part of the GenEMBL database, where it was present as multiple submissions of a known gene sequence (18,22, and references therein). The peptide sequence of yCBP80 shows ~33% similarity to human CBP80.

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yCBP20 was characterized independently in two studies. Görlich *et al.* (22) found a significant amount of yCBC in association with SRP1p (23,24), a subunit of the yeast nuclear protein import receptor (25). This observation may have implications for the role of CBC in RNA export and there is evidence that the vertebrate homologue of the yCBC–Srp1p complex may be a functional intermediate in the cycling of CBC between the nucleus and cytoplasm (22). Purified yCBC shows a similar, but not identical, cap binding specificity to the human complex. In parallel, the yeast *MUD13* gene was found in a genetic screen which was designed to identify components of the commitment complex (26) and shown to encode the yeast homologue of CBP20 (27). It was shown that *mud13* mutant strains were deficient for pre-mRNA splicing both *in vivo* and *in vitro* (27).

In this report we demonstrate that immunodepletion of yCBC from yeast *in vitro* splicing extracts results in inefficient assembly of both commitment complexes and spliceosomes, as well as to a reduction in pre-mRNA splicing. These defects can be partially restored by the re-addition of yCBC. Our data are in good agreement with, and complementary to, data obtained by Colot *et al.* (27), who show that extracts prepared from a *mud13* mutant strain, and thus lacking yCBP20, are defective for both commitment complex assembly and splicing. Taken together with the data from human cell extracts of Lewis *et al.* (21), these data show that the function of CBC in promoting early steps in recognition of pre-mRNA is conserved between yeast and mammals.

MATERIALS AND METHODS

DNA constructs and *in vitro* transcription

Plasmids pBS195 (28) and SP6-actin (29) were a kind gift from Dr Bertrand Séraphin. pBS195 was linearized with *Dde*I and transcribed by T7 RNA polymerase and SP6-actin was linearized with *Msp*I and transcribed by SP6 RNA polymerase. Transcriptions used 0.5 µg cleaved DNA as template with nucleotides at 500 µM, except for UTP, which was at 50 µM, and 1 mM m^7GpppG with 40 µCi [α-^{32}P]UTP (Amersham) in the reaction using Promega enzymes and buffers.

Splicing extract preparation

Splicing extracts were made as described by Lin *et al.* (30), but were not dialysed prior to immunodepletion. After immunodepletion they were dialysed into buffer D50 (50 mM KCl, 20 mM HEPES, pH 7.5, 20% glycerol, 1 mM DTT) and flash frozen in liquid nitrogen.

Native gel analysis of commitment complex and spliceosome formation

Native gel analysis was as described by Séraphin and Rosbash (5). All native gels were prepared with glycerol at a final concentration of 5%.

In vitro splicing

Splicing reactions (30) were incubated for 40 min at 25°C and used m^7GpppG capped substrates (29). For splicing add-back reactions, equivalent volumes of yCBC dialysis buffer (20 mM Tris, pH 7.5, 250 mM sucrose, 50 mM NaCl, 5% glycerol) were added to each reaction to control for non-specific buffer effects. Spliced products were recovered and resolved by 8% denaturing PAGE.

Preparation of yCBC

A 1 ml column of affinity-purified immobilized rabbit antibodies raised against a peptide consisting of the N-terminal 10 amino acids of yCBP80 (22) was loaded with yeast high speed supernatant, prepared as described (22) and adjusted to 50 mM HEPES–KOH, pH 7.5, 50 mM Tris–HCl, pH 7.5, 50 mM potassium acetate, 200 mM NaCl, 5 mM β-mercaptoethanol, 10 mg/ml leupeptin, 5 mg/ml each of chymostatin and elastitinal, 10% glycerol. Bound material was eluted with 1 mg/ml antigenic peptide in 50 mM Tris, pH 7.5, 1 M NaCl at room temperature. Peak fractions were pooled and further purified on a Superdex 200 column equilibrated in 50 mM Tris, pH 7.5, 100 mM NaCl, 3 mM mercaptoethanol. Peak fractions, identified first by protein content and then by SDS–PAGE analysis, were dialysed into 20 mM Tris, pH 7.5, 250 mM sucrose, 50 mM NaCl, 5% glycerol for adding back to depleted extracts.

Immunodepletion of splicing extracts

Immobilized immunopurified anti-yCBP80 antibodies raised against recombinant yCBP80 (22) or, as a control, a 1:1 mix of protein A and protein G was pre-washed with an equal volume of yeast splicing extract. For depletion, the ratio of extract to beads was 3:1. Extracts were depleted in batch with gentle rotation for 90 min. The beads were then pelleted at 2000 *g* for 1 min and the supernatant recovered and stored on ice. The immobilized antibodies were regenerated by washing twice with 10 vol 100 mM glycine, pH 3, and the beads were then equilibrated in buffer D250 (250 mM KCl, 20 mM Tris, pH 7.5, 20% glycerol, 1 mM DTT), pelleted and the supernatant aspirated off. The beads were then washed once with 0.5 vol either depleted or mock depleted extract and a second round of depletion done for 90 min. The supernatants were then dialysed against buffer D50. Depletion was checked by Western blotting. Extracts were resolved by 12.5% SDS–PAGE, blotted onto nitrocellulose and the filter blocked in 1× PBS, 5% dried milk, 1% Triton X-100. The blot was then probed using either anti-yCBP80 or anti-yCBP20 antibodies at 1:5000 dilution in 1× PBS, 1% dried milk, 1% Triton-X100 for 45 min then washed twice with a large volume of buffer. The secondary antibody was used at a dilution of 1:1000 in the same buffer as above. The blot was developed using ECL (Amersham International).

RESULTS

Purification of yCBC

Functional yCBC was purified from yeast extracts using an antibody raised against an N-terminal peptide of yCBP80 (22). The peptide-eluted material (Fig. 1, left panel) shows a very high enrichment in the subunits of yCBC (yCBP80 and yCBP20) as determined by sequence analysis of the two proteins and by cap-specific binding in a gel mobility shift assay (22). Further purification by size exclusion chromatography, in addition to demonstrating that yCBP80 and yCBP20 co-fractionate, results in fractions containing an almost homogenous preparation of yCBC (Fig. 1, right panel).

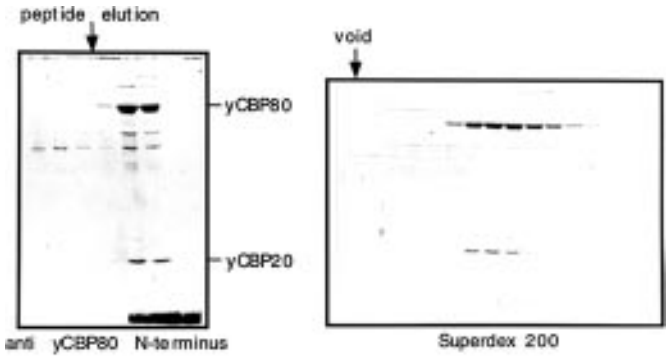

Figure 1. Purification of yCBC. High speed extracts from *S.cerevisiae* were loaded onto an antibody column to which antibodies against an N-terminal peptide of yCBP80 were bound (see Materials and Methods) and eluted by the antigenic peptide (22). The left panel of the figure shows the last three wash fractions (left hand lanes) and the first four fractions after peptide addition to the column. Aliquots of these fractions were separated by SDS–PAGE and Coomassie stained. The eluted material was dialysed and further purified by size fractionation on a Sephadex 200 column. Aliquots of peak fractions, identified by protein assay, were fractionated by SDS–PAGE and Coomassie stained. The positions of yCBP80 and yCBP20 are indicated.

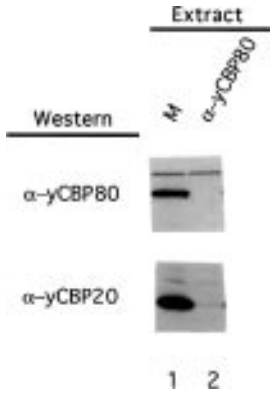

Figure 2. yCBP20 is efficiently co-immunodepleted from splicing extracts by anti-yCBP80 antibodies. Splicing extracts were depleted using immobilized anti-yCBP80 antibodies. Depletion was assayed by Western blot using either anti-yCBP20 or anti-yCBP80 antiserum, comparing depleted extract to mock depleted extract as indicated.

yCBC is required for efficient commitment complex and spliceosome formation

In mammalian splicing extracts, CBC is required to promote efficient association of U1 snRNP with the 5′-splice site (21). In order to determine whether the function of yCBC in pre-mRNA recognition was conserved, yeast splicing extracts were immunodepleted using immobilized antiserum raised against recombinant yCBP80 (see Materials and Methods). The levels of depletion were assayed by Western blot using antiserum raised against either yCBP80 or yCBP20. As can be seen in Figure 2, yCBP80 is efficiently depleted from the extract [compare mock depleted (M) lane 1 with depleted (D) lane 2]. yCBP20 is efficiently co-depleted, as would be expected from its co-fractionation with yCBP80 (compare lanes 1 and 2), reinforcing the conclusion that, as is the case in human cell extracts (18), the greater parts of yCBP80 and yCBP20 are present as a complex.

The extracts depleted of yCBC were assayed for their ability to form commitment complexes. Uniformly labelled wild-type pre-mRNA was synthesized, primed with a m^7GpppG cap. Complexes were allowed to form on the pre-mRNA in the absence of ATP and then resolved on a native agarose–polyacrylamide gel (see Materials and Methods). As can be seen in Figure 3, depletion of yCBC leads to a marked reduction in the level of commitment complex (both CC1 and CC2) observed (compare lanes 2 and 5; note that in these extracts CC1 is present only in minor quantities). The residual complex which forms in the depleted extract migrates on the gel with a lower mobility than normal CC2. Although we do not know the explanation for this behaviour, Colot *et al.* (27) also observed that commitment complexes formed in extract lacking yCBP20 migrated more slowly than complexes formed in wild-type extract.

To determine whether the effect of yCBC was specific and reversible we added back yCBC purified close to homogeneity (Fig. 1) to the depleted extract to examine whether it could restore commitment complex formation. When increasing amounts of purified yCBC were added to the depleted extract, CC1 and CC2

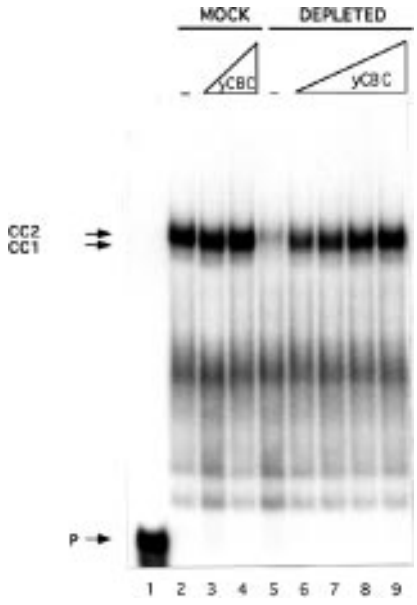

Figure 3. Purified yCBC can restore commitment complex formation to depleted extracts. Purified yCBC was added to depleted extract lanes (lanes 6–9) or to mock depleted extract at the lowest and highest amounts (lanes 3 and 4). Complex assembly was allowed to proceed for 15 min (described in Materials and Methods) and complexes were resolved by native gel electrophoresis (5). The positions of commitment complexes (CC1 and CC2) and free probe (P) are indicated by arrows.

formation was restored to a level ~50% that of mock depleted extract (compare lane 5 with lanes 6–9). Attempts to further concentrate the purified yCBC and retain activity were unsuccessful and we were therefore unable to determine whether the incomplete rescue is due to re-addition of insufficient amounts of the complex or to partial co-immunodepletion of a second factor involved in CC1 and CC2 formation with yCBC. Stimulation of CC1 and CC2 assembly was observed with the highest amount of yCBC added to the mock depleted extract (lane 4), suggesting that the concentration of yCBC may be limiting for commitment complex formation in these mock depleted extracts.

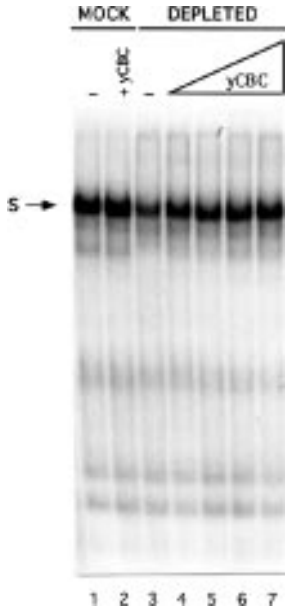

Figure 4. Purified yCBC partially restores spliceosome formation to depleted extracts. Spliceosome formation was assayed in the presence of ATP. Increasing amounts of purified yCBC were added to depleted extracts (lanes 4–7) and, as a control, the highest amount only was added to the mock depleted extract (lane 2). In lanes 1 and 3 an equivalent volume of buffer was added to the reaction. The reactions were incubated for 20 min then spliceosomes (S) were resolved from free probe by native gel electrophoresis (5).

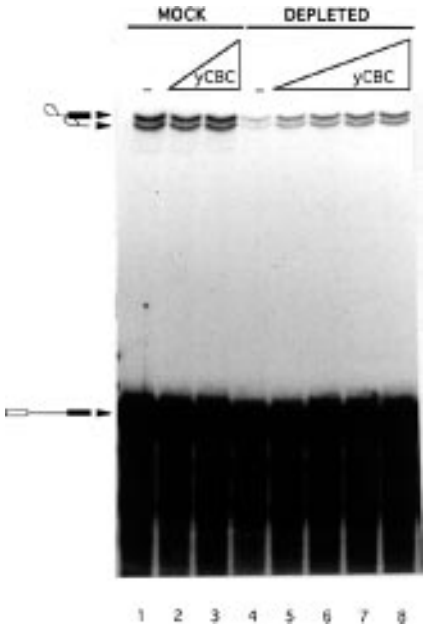

Figure 5. yCBC is required for efficient pre-mRNA splicing *in vitro*. Mock or depleted extracts were assayed for their ability to splice m^7GpppG capped actin pre-mRNA (lanes 1 and 4). Increasing amounts of purified yCBC were added to the depleted extract (lanes 5–8). As a control, the lowest and highest amounts of yCBC was added to the mock depleted extract (lanes 2 and 3). The RNA products were recovered and resolved by denaturing PAGE.

Since spliceosome assembly follows a linear stepwise progression, a defect in commitment complex assembly should cause a subsequent reduction in spliceosome accumulation. To assay spliceosome complex formation, similar reactions to those above, but in the presence of exogenous ATP, were carried out. Figure 4 shows that spliceosome assembly is inhibited by ~70–80% in depleted versus mock depleted extracts (lanes 1 and 3). In order to determine whether this effect was reversible, increasing amounts of yCBC were added to the depleted extract. Spliceosome formation can be restored to ~50% of the mock depleted control (lanes 4–7). Addition of the highest amount of yCBC to the mock depleted extract did not greatly stimulate the level of spliceosome formation (compare lanes 1 and 2).

yCBC is required for efficient pre-mRNA splicing *in vitro*

In mammalian nuclear extracts, depletion of CBC results in inhibition of the first step of pre-mRNA splicing by ~90%. We were interested to determine whether this was also paralleled in the yeast splicing extract depleted of yCBC. Uniformly labelled m^7GpppG-capped actin pre-mRNA was therefore added to either mock depleted or yCBC depleted extracts. Depletion of yCBC resulted in a strong inhibition of pre-mRNA splicing (Fig. 5, lanes 1 and 4). Quantitation of this and other experiments has shown that the inhibition observed was routinely between 70 and 80%. Splicing could be restored to the depleted extracts by addition of increasing amounts of purified yCBC (lanes 5–8) to a level similar to the restoration of spliceosome formation (see above). No general stimulation of splicing was observed upon addition of yCBC to the mock depleted control reactions.

DISCUSSION

The experiments presented here show that yCBC is required for efficient commitment complex assembly, spliceosome assembly and pre-mRNA splicing *in vitro*. The inhibition of commitment complex formation observed in depleted extracts leads to a strong reduction in spliceosome formation and a reduction in the levels of splicing observed. These data complement the work by Colot *et al.* (27), who showed not only that mutation or deletion of the *MUD13* gene, which encodes Mud13p, the yeast homologue of CBP20, affected splicing efficiency *in vivo*, but also that extracts prepared from *mud13* mutant strains show impaired ability to form commitment complex (both CC1 and CC2) and to carry out pre-mRNA splicing. Interestingly, if yCBC is removed either by immunodepletion (this paper) or genetically by disruption of the *MUD13* gene (27), the small amount of commitment complex which is formed has a lower mobility than the wild-type commitment complex. Since CBC appears to be a component of CC1 and CC2 (27), one might have expected the opposite result, i.e. that complexes formed in extracts lacking CBC would exhibit a higher mobility. This suggests that the presence of CBC in the commitment complex may, either directly or indirectly, induce a conformational change that results in a more compact RNP. Addition of yCBC to depleted extract both increases the quantity of commitment complexes formed to ~50% of that seen in mock depleted extract and restores the mobility of these complexes to that observed in wild-type extracts, demonstrating that both changes are specific results of the lack of CBC.

As would be expected, the defect observed in commitment complex formation in the absence of yCBC is reflected in deficiencies in both the formation of spliceosomes and in

pre-mRNA splicing. The effects of yCBC depletion in each case could be partially (to a level of ~50%) restored by the re-addition of highly purified yCBC. There are two possible reasons for the incompleteness of the recovery of activity. The first is that we may be unable to add sufficient yCBC to completely regain activity. This is suggested by the facts that yCBC is probably rate limiting for CC1 and CC2 formation in our extracts (Fig. 3) and that progressive increases in restoration of activity were seen in all three assays up to the maximal levels of yCBC added back. Unfortunately, for technical reasons, we were unable to obtain active preparations of yCBC at higher concentrations. The second possible reason is that we may partially co-deplete a second factor involved in commitment complex formation, such that this factor becomes limiting when enough yCBC is added to the depleted extract. As previously mentioned, a fraction of up to ~30% of yCBC is associated with Srp1p, the yeast nuclear protein import receptor (22). However, immunodepletion of Srp1p from splicing extract had no detectable effect on splicing (data not shown), indicating that this trimeric complex does not have a specific function in splicing.

Functional conservation of CBC function in pre-mRNA splicing

The overall conservation of the amino acid sequence of CBP80 and CBP20 between humans and *S.cerevisiae* (18,22,27) and other higher eukaryotes (our unpublished data) would suggest that the function of CBC should be conserved. Taken together, the evidence from both yeast (this paper and 27) and mammalian systems (21) strongly supports the model that CBC binding to the cap structure of a pre-mRNA facilitates an early step in splicing complex assembly, most likely U1 snRNP association with the 5′-splice site. Although this does not explain how CBC facilitates U1 snRNP association with the 5′-splice site, it does suggest that the mechanism will be conserved. Further work will be required to determine whether yCBC functions by directly interacting with a known component of the commitment complex, like the U1 snRNP, or whether additional factors are required to mediate the effect of yCBC on complex formation.

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