Boric acid reversibly inhibits the second step of pre-mRNA splicing

Noam Shomron, Gil Ast*

Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Tel Aviv, Israel

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Abstract Several approaches have been used to identify the factors involved in mRNA splicing. None of them, however, comprises a straightforward reversible method for inhibiting the second step of splicing using an external reagent other than a chelator. This investigation demonstrates that the addition of boric acid to an in vitro pre-mRNA splicing reaction causes a dose-dependent reversible inhibition effect on the second step of splicing. The mechanism of action does not involve chelation of several metal ions; hindrance of 3′ splice-site; or binding to hSlu7. This study presents a novel method for specific reversible inhibition of the second step of pre-mRNA splicing.

Key words: Pre-mRNA splicing; Inhibition; Boric acid; Spliceosome

1. Introduction

The spliceosome, a multicomponent complex of proteins and RNA, is assembled on the newly synthesized precursor messenger RNA (pre-mRNA) to catalyze a two-step transesterification reaction required to remove the introns and ligate the exons (reviewed in [1]). The assembly of the spliceosome proceeds through a coordinated assembly of complexes (represented by the letters E, A, B, C and I) or, alternatively, as recently reported, through a one-step assembly [2–4]. More than 140 proteins are involved in the splicing reaction [5], some 15 of which are associated with purified complex C in which splicing is arrested after the first step [6]. Currently, six proteins have been found necessary for the second step of splicing in yeast, and their human homologs have been identified (reviewed in [5]). The involvement of these and other factors in the second step is still far from understood.

The present study is an in vitro investigation of the effects of boric acid on pre-mRNA splicing. Humans are exposed to high concentrations of boric acid through pesticides, cosmetics and medicines [7–9]. At high consumption boric acid is toxic to all species through the reproductive and developmental systems [7], though it is not carcinogenic or mutagenic [10]. Boric acid does not appear to be metabolized in humans or animals [11] and at low consumption it is reported to aid in wound healing possibly by increasing RNA synthesis of tumor necrosis factor α [12], vascular endothelial growth factor and transforming growth factor β [13]. Recent studies suggest that men who consume high levels of boron (found in fruits and nuts) reduce their risk of prostate cancer [14] possibly by enzymatic inhibition [15]. Borates can form a reversible borate–ester complex through intramolecular hydrogen bonding at the nucleoside sugars' *cis*-2′,3′-diol, which can interfere with base pairing interactions [16]. In addition, borates can disrupt protein activities [17] using two possible reversible mechanisms; the first involves a covalent boronic acid tetrahedral adduct formed with the serine oxygen at the active site of the enzyme that mimics a high-energy transition state structure [18]; the second involves inhibition of metalloenzymes by binding to metal ions in active sites and forming a stable transition state analog [19].

We demonstrate that the addition of boric acid to an in vitro mRNA splicing reaction efficiently and specifically inhibits the second step of splicing, and is accompanied by the buildup of active spliceosomes paused before completion of step two of the splicing reaction. The effect of boric acid is reversible and is probably induced by the attachment of three oxygen atoms to the boron atom. We conclude that the mechanism of action does not involve the chelation of several metal ions; hindrance of the 3′ splice site (SS); or binding to a second step splicing metalloprotein, hSlu7. This study presents a novel method for specific reversible inhibition of the second step of pre-mRNA splicing.

2. Materials and methods

2.1. Splicing substrates, reactions, and spliceosome complex analysis

Standard splicing reactions were performed as described [20]. Briefly, uniformly labeled RNA transcript was incubated in in vitro splicing conditions with the addition of boric acid, butylboronic acid, methylboronic acid, 3-aminophenylboronic acid, borax (sodium borate) and trimethyl borate, all brought to pH 7.9. To some of the reactions divalent metal cations were added while to others recombinant hSlu7 protein was added (see below). RNA was extracted and analyzed by 8% denaturing polyacrylamide gel electrophoresis as described [21]. Spliceosome complexes were analyzed in 4% native gels as described [22] (with 0.5 mg/ml heparin in the loading buffer). The dialysis procedure was performed as described [20].

2.2. Recombinant hSlu7 purification

The hSlu7 cDNA was provided by R. Reed and cloned into pGEX-6P expression vector (Amersham-Pharmacia) fused upstream of the glutathione *S*-transferase (GST) sequence. The recombinant hSlu7-GST protein was expressed in *Escherichia coli* BL21 (DE3) strain (Stratagene). Briefly, following induction with 0.5 mM isopropyl-β-D-thiogalactose for 6 h at 25°C cells were lysed by sonication in lysis buffer (phosphate-buffered saline, 50 mM EDTA, 1% Triton), cell debris was removed by centrifugation, and hSlu7-GST fused protein was captured using glutathione Sepharose 4 Fast Flow as described by the manufacturer (Amersham-Pharmacia). Recombinant protein was purified to more than 95% homogeneity.

2.3. Site-specific labeling of the pre-mRNA

Site-specifically labeled pre-mRNA at position −1 of the 3′ SS (the

*Corresponding author. Fax: (972)-3-640 9900.
E-mail address: gilast@post.tau.ac.il (G. Ast).

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G nucleotide) was generated by transcribing two RNA templates: the first from the 5′ exon to position −2 of the 3′ SS (the A nucleotide), and the second from position −1 of the 3′ SS (the G nucleotide) to the 3′ exon. The 3′ segment was dephosphorylated with 1 U of alkaline phosphatase (Roche), end-labeled in a 20 μl reaction mixture containing 3.0 μl of [γ-^{32}P]ATP (3000 Ci/mmol), 2 U of T4 polynucleotide kinase (New England Biolabs), and 4 μl of the supplied buffer from the same manufacturer. The 5′ and 3′ segments were ligated in a 20 μl reaction mixture with a DNA oligonucleotide bridge spanning both RNA fragments according to the manual (TaKaRa ligation kit). The ligated product was separated in a 5% denaturing gel and extracted for further experiments.

3. Results

3.1. Boric acid inhibits the second step of splicing

To examine the effect of boric acid on in vitro mRNA splicing, HeLa nuclear extract was incubated under in vitro conditions for 60 min with a labeled human β-globin transcript, the RNA was then extracted and separated in a denaturing gel. Addition of 5 mM boric acid led to an increase in the amount of splicing intermediates and products (Fig. 1A, compare lanes 1 and 2; an average two-fold increase in splicing activity was quantitated in five different experiments; data not shown; also see Section 4). However, only the products of the second step of the splicing reaction decreased considerably when 18 mM boric acid was added while first step products were maintained at a high level (Fig. 1A, lane 4). An almost complete inhibition of splicing was observed when 22 mM of boric acid was added to the splicing reaction, followed by a complete arrest of splicing activity at 26 mM (Fig. 1A, lanes 5 and 6, respectively). We concluded that the addition of 18 mM boric acid efficiently and specifically inhibited the second step of splicing.

The inhibition of the second step of splicing by boric acid was observed with six different nuclear extracts. The specific inhibiting concentration of boric acid differed from one extract to the other but stayed within a 12–20-mM range (see Fig. 3 for an additional example). The observation that different nuclear extracts have different ranges of second step splicing inhibition suggests that they harbor different concentrations of an affected factor(s). Thus, it is important to individually titrate each nuclear extract in order to identify its inhibitory concentration. Also, the second step splicing inhibition by boric acid is a general phenomenon as five different mRNA precursors exhibited a similar inhibition (data not shown).

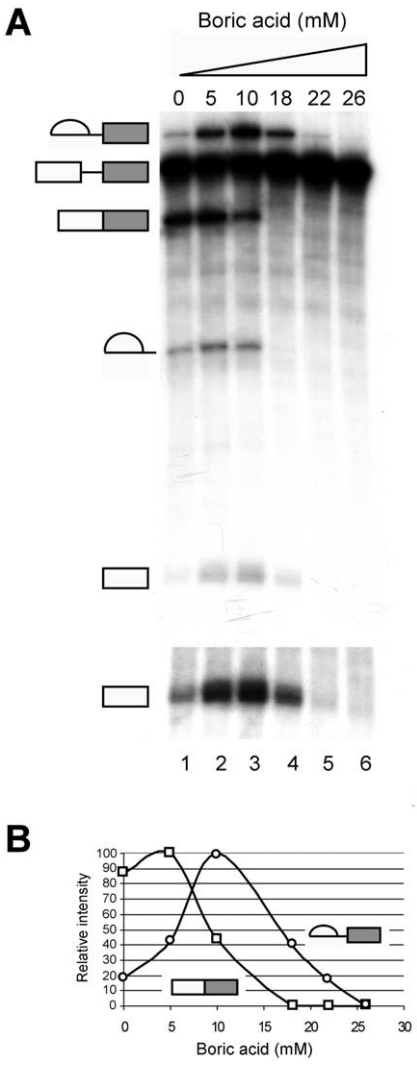

Fig. 1. Boric acid can efficiently inhibit the second step of mRNA splicing. A: A radiolabeled transcript of β-globin was incubated in an in vitro splicing reaction. The indicated concentration of boric acid (see Fig. 3A for chemical structure) was added to each reaction and the reactions were incubated for 60 min at 30°C. RNA was extracted and separated in an 8% denaturing gel. RNA intermediates and products are schematically represented on the left. Exons are drawn as boxes and introns as lines. A longer exposure of the free 5′ exon is added below the main gel. B: Quantification of first (circles) and second step (squares) splicing products presented in A.

3.2. The effect of boric acid on the spliceosome

To investigate the effect of boric acid on spliceosome assembly, we analyzed the complexes formed in the presence of increasing boric acid concentrations. HeLa nuclear extract was incubated with a radiolabeled β-globin transcript for 60 min under splicing conditions and the splicing reaction was then separated in 4% native polyacrylamide gels (Fig. 2). In the absence of boric acid, complex A was detected after 5 min of incubation and declined thereafter. Complexes B and C appeared after 5 min, reached a peak at 15 min, and declined steadily thereafter. Thus, after 60 and 90 min of incubation A, B and C complexes were barely detectable (see also [20,22]).

The addition of boric acid at 5 and 10 mM was accompanied by increasing accumulation of complexes B/C (Fig. 2A, lanes 2 and 3), reaching their peak at 18 mM (Fig. 2A, lane 4) and declining thereafter at 22 and 26 mM (Fig. 2A, lanes 5 and 6). These complexes also were maintained even at longer incubation periods (data not shown). The RNA content within complexes B/C was determined showing first step splicing intermediates only (data not shown; see also [20]). In the absence of boric acid a newly identified complex, termed M, was detected after 15 min incubation (Fig. 2B). This complex contained RNA products of the second step of splicing evidenced by extraction of the RNA from complex M and analysis in a denaturing gel (Fig. 2C; also see Section 4). Panel D is a quantification of complexes A and B/C as a function of boric acid concentration, indicating the accumulation of active spliceosomes (B/C) at 18 mM concentration. Thus, the presence of boric acid at a range of concentrations that inhibits the second step of splicing was accompanied by buildup of complexes B/C and inhibition of a unique complex M.

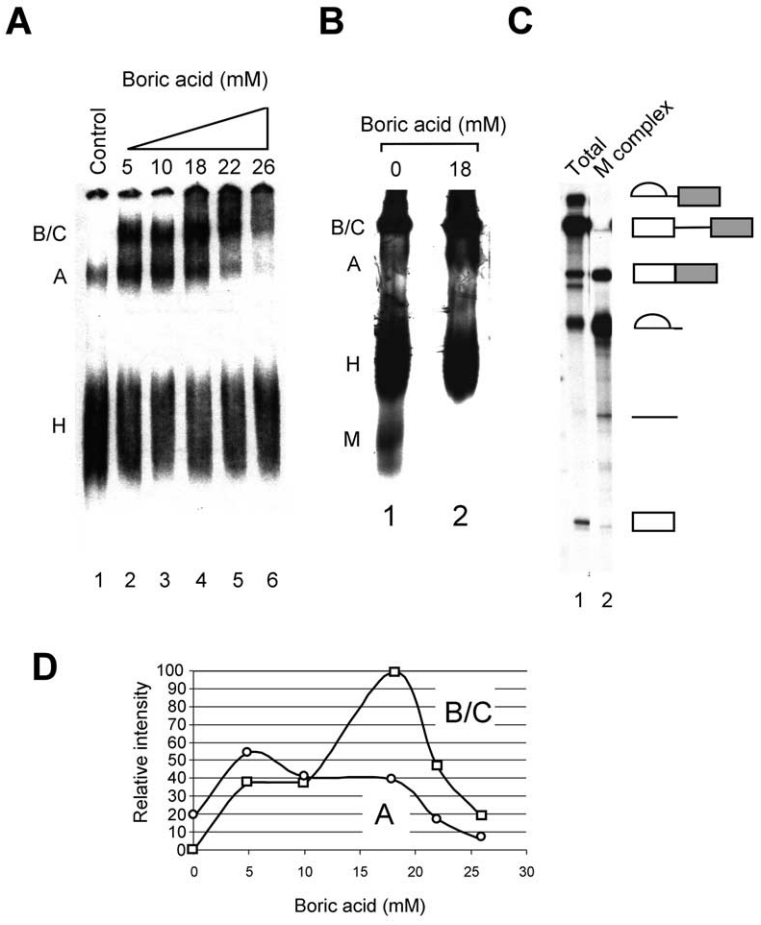

Fig. 2. The effect of boric acid on the spliceosome. A: A radiolabeled β-globin transcript was incubated in nuclear extract under splicing conditions in the presence of the indicated concentrations of boric acid. Splicing complexes were fractionated in a 4% non-denaturing gel and complexes were identified according to their respective location on the gel and analysis of their labeled mRNA contents (data not shown). B: A similar experiment was performed as described in A, but this time the reaction was carried out for 15 min. C: Analysis of the labeled mRNA contents within complex M. Complex M, marked in panel B, was eluted from the gel and the labeled RNA was extracted and separated in 8% denaturing gels. Unfractionated splicing reaction (Total) was run next to products extracted from complex M. RNA intermediates and products are schematically represented to the right of the gel. There was no labeled RNA within the corresponding area of complex M in the reaction incubated in the presence of boric acid (data not shown). D: Quantification of A (circles) and B/C (squares) complexes presented in panel A.

3.3. The inhibition is specific to boric acid

The inhibition of the second step of splicing was specific to boron attached to three oxygen atoms since boronic acid compounds, methyl- and butylboronic acid (see Fig. 3A for chemical structures), did not inhibit splicing even at 22 and 30 mM (Fig. 3B, compare BA to MBA and BBA). In addition, two different boronic acid compounds that are known to dissociate to boric acid in aqueous solution, trimethyl borate and borax, exhibited a similar inhibition to boric acid (data not shown). 3-Aminophenylboronic acid (see Fig. 3A, APBA), which binds *cis*-diol groups with high affinity, inhibited the first step of splicing at 18 mM thus suggesting that it acts on RNA itself, and strengthening the assumption that boric acid exerts its effect in a different manner. We could not exclude, however, the formation of a boric ester complex with a terminal ribose of the free 5′ exon.

3.4. The inhibition effect is reversible

To test whether the inhibitory effect on the second step of splicing is reversible we dialyzed the boric acid from the splicing reaction. Splicing reactions were incubated for 60 min in the absence or presence of 18 mM boric acid, and one part of each reaction was removed while the other part was dialyzed for 12 h against the nuclear extract buffer. The reactions were then removed from the dialysis tube, a new set of co-factors was added, and both the undialyzed and dialyzed reactions were further incubated for 60 min (Fig. 4A, lanes 1–3 and 4–6, respectively). The inhibition effect on the second step of splicing was reversible following 12 h dialysis (Fig. 4A, compare lanes 2 and 5). When 26 mM boric acid was used there was only a partial reversibility suggesting that at these concentrations another factor(s) might be affected by boric acid which is not reversible after dialysis (one, for example, that binds covalently to boric acid; Fig. 4A, compare lanes 3 and 6).

We examined this further by showing that the splicing-inhibited reaction, dialyzed for not more than 3 h, restores only the first step of splicing, even in the presence of an unlabeled transcript competitor (at 500 times molar excess in order to eliminate re-assembly of new spliceosomal complexes on the labeled transcript following the dialysis [23,24]; data not shown).

This finding further suggested that the inhibition of the second step was not due to the interaction of boric acid with the 3′ end hydroxyl group of the first exon following

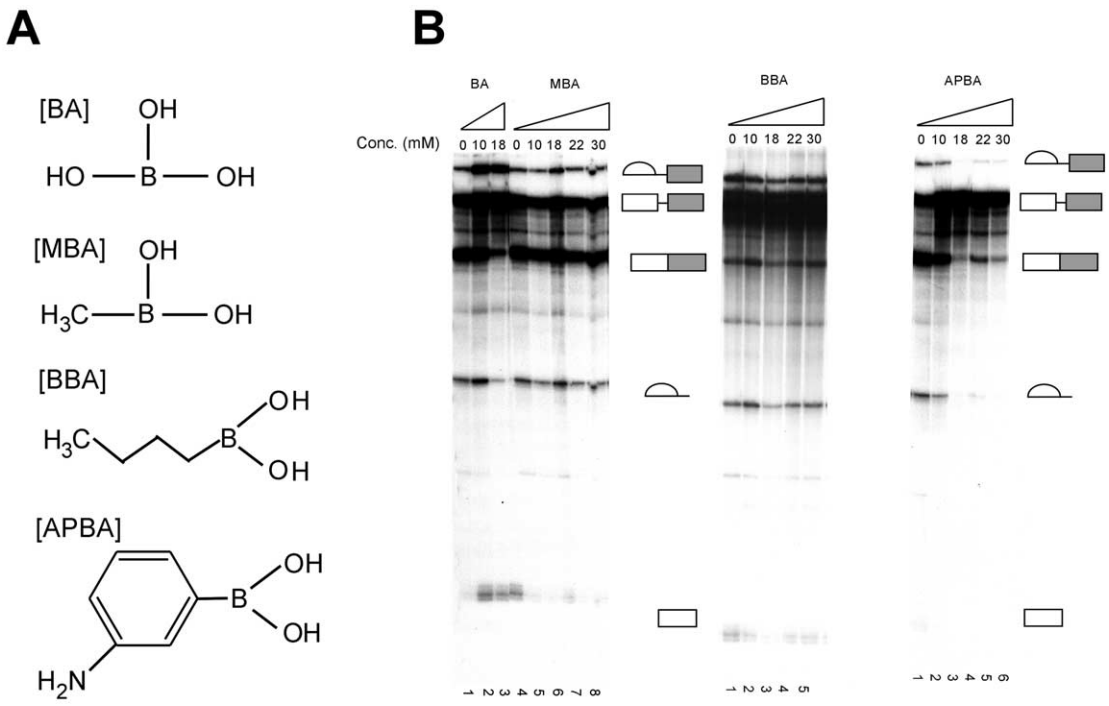

Fig. 3. The effect of various boronic acid compounds on splicing activity. A: The chemical structure of each compound used: BA, boric acid; MBA, methylboronic acid; BBA, butylboronic acid; APBA, 3-aminophenylboronic acid. B: A radiolabeled β-globin transcript was incubated in nuclear extract under standard splicing conditions in the presence of the indicated concentrations of the above reagents. Following 60 min incubation at 30°C, RNA was extracted and separated in an 8% denaturing gel.

the first step of splicing. Thus, we conclude that boric acid can be used as an efficient reagent to inhibit the second step of splicing, and the inhibition can be reversed by a 12-h dialysis process.

To eliminate the possibilities that we titrated splicing cofactors or that boric acid interacted electrostatically with the RNA, we added ATP, $MgCl_2$, creatine phosphate and sucrose (which has a 2′,3′-diol group) to the splicing-inhibited reaction. The inhibition effect could not be released by any of these reagents suggesting that the inhibition effect of boric acid is not a simple interaction of the boric acid with the diol groups on the ribose (as in [16,25–27]) nor a saturation of the hydroxyl residues of boric acid (as in [28]).

3.5. *The inhibition mechanism is not due to the chelation of Cu, Mn, or Zn, binding to hSlu7 or 3′ SS hindrance*

There were indications that boric acid did not lead to formation of non-functional spliceosomes: it did not interfere with the normal assembly pathway of spliceosomal complexes (A and B/C; Fig. 2) and splicing activity was restored following the dialysis experiment, in particular in the presence of a competitor (Fig. 4A and data not shown). To further test the possibility that boric acid induces inter-spliceosomal interference, we used a site-specific ^{32}P-labeled RNA transcript (at position −1 of the 3′ SS) that was incubated in in vitro splicing conditions in the absence or presence of inhibitory boric acid concentrations. Although no attempt was made to identify the crosslinked proteins, a comparison of those incubated in the absence and presence of boric acid revealed no significant differences (Fig. 4B). This implies that boric acid did not induce a major interference in the binding of proteins to the 3′ SS. Moreover, there was no difference in the binding of U1 and U2 snRNA to the 5′ SS and branch site sequences, respectively, as examined by crosslinking with psoralen (data not shown).

We have previously shown that chelation of zinc from the splicing reaction pauses splicing after the first step [20]. In order to test whether boric acid and zinc function in a similar manner, we performed an add-back reaction to try to reverse the inhibitory effect of boric acid. However, none of the divalent metal ions used in our experiments, copper, manganese and zinc, was able to restore the inhibition of the second step, suggesting that zinc and boric acid affect mRNA splicing in different manners (data not shown).

Currently, only one of the known second step splicing proteins, hSlu7, contains a potential zinc knuckle domain and a crosslinked protein with a similar molecular weight to hSlu7 (MW 68 kDa) was identified in that region [29]. The p68 crosslinked protein might be slightly affected by incubation in the presence of boric acid (Fig. 4B, indicated by an arrow). We thus set out to determine whether addition of boric acid disrupted hSlu7's binding to the 3′ SS. We reasoned that if boric acid inhibits hSlu7's function by binding to its metal ion, addition of excess protein to the reaction (at almost equal molar concentrations to the boric acid) will alleviate the inhibition. However, purified recombinant hSlu7 (proved to be active in mRNA splicing; data not shown) could not release the inhibition, suggesting that hSlu7 is not the splicing factor affected by boric acid (data not shown).

4. Discussion

We have developed a simple method using boric acid to specifically and efficiently inhibit the second step of mRNA splicing. This inhibition is reversible upon prolonged dialysis, and the inhibitory effect requires the boron atom to be at-

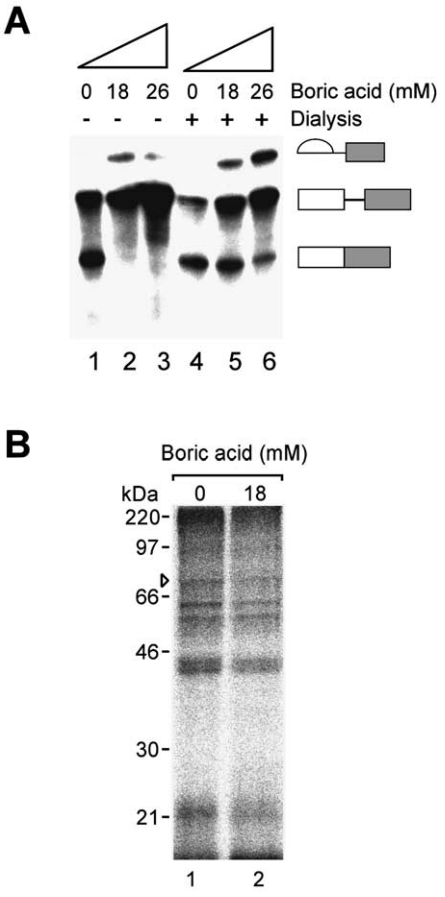

Fig. 4. Relieving the inhibitory effect of boric acid on the second step of splicing. A: A radiolabeled β-globin transcript was incubated in nuclear extract under standard splicing conditions in the absence or presence of the indicated concentration of boric acid. Following 60 min incubation at 30°C, one part of the reaction was removed (lanes 1–3). The other part was dialyzed against the nuclear extract buffer [36] for 12 h, after which another set of co-factors (ATP, creatine phosphate and $MgCl_2$) was added (lanes 4–6). Both the dialyzed and undialyzed reactions were further incubated for another 60 min at 30°C. RNA was purified and separated in an 8% denaturing gel. B: Boric acid does not hinder the 3′ SS. A site-specific ^{32}P-labeled RNA transcript (at position −1 of the 3′ SS) was incubated in in vitro splicing conditions in the absence or presence of inhibitory boric acid concentrations. Following 30 min incubation at 30°C, the reaction was crosslinked at 254 nm, treated with RNase T1, and the proteins were separated in a 10% SDS-protein gel.

tached to three oxygen atoms. To the best of our knowledge, this is the first mRNA splicing-related effect associated with borate. The inhibition of the second step of mRNA splicing by boric acid can be used to identify the factors and the network of interactions involved in this step. Given that inhibition concentrations ranged from 12 to 20 mM in our six different nuclear extracts, it is recommended to titrate the nuclear extract to identify its inhibitory concentration.

Our results indicate the resemblance between the unique M complex, first identified here, located below complex H on the native gel (Fig. 2B), and the 15S complex of the glycerol gradient [30]. Analysis of complexes separated in the native gels and mRNA products within this complex (Fig. 2C) led us to conclude that the inhibition of the second splicing step coincided with the accumulation of B/C complexes and inhibition of complex M formation. It is worth noting that β-globin pre-mRNA alone ran out of the native gel at these conditions, and that the snRNPs ran above complex M, except for U1 which ran at the bottom of the gel due to the treatment of the splicing reaction with heparin (data not shown). This suggests that complex M represents RNA products assembled with proteins and without snRNPs. However, we cannot distinguish at this point whether the mRNA and the intron lariat within the M complex are two different complexes that co-migrate, or the same complex.

It is intriguing that the inhibitory effect of boric acid on the splicing reaction commences at millimolar concentrations, which is greatly in excess of the spliceosomal component's concentration [31]. Nevertheless, this inhibition is rather specific since boronic acid compounds without a three-hydroxyl group surrounding the boron atom eliminated the inhibitory effect. Similarly, specific inhibitions of enzymatic reactions by borates at concentrations between 10 and 50 mM have been demonstrated elsewhere [15,32–35]. Alternatively, if boric acid interacts with many factors in the nuclear extract, higher concentrations are required to affect the factor(s) involved in the second step of splicing.

The hSlu7 protein is currently the only known metalloprotein involved solely in the second step of splicing, yet we cannot exclude that other splicing metalloproteins were affected by the addition of boric acid. This argument is supported, at least at the 26 mM concentration, by its partial reversibility following dialysis.

At low concentrations of boric acid, usually 5–10 mM, an increase in splicing activity is observed. It was previously reported that 10 mM boric acid increases in vitro RNA synthesis by more than 10-fold [13]. The increase in splicing in our experiments is more of a general phenomenon and acts on all transcripts tested. We cannot exclude that during in vitro splicing boric acid protects the RNA from degradation. It is recommended that 5 mM of boric acid be added to the splicing in vitro reaction to improve splicing activity.

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