haemagglutinin (HA) and six-histidine tags. pST1420 contained the mutant *cut14* gene.

Overproduction and protein purification. For overproduction of Cut3 and Cut14 proteins, cells carrying the plasmids described above were cultured in minimal EMM2 in the absence of thiamine for 14 h at 33 °C (for overproducing the mutant Cut14 protein, for 23 h at 26 °C). Whole-cell extracts were prepared for $1-2 \times 10^{10}$ cells. Subsequent manipulations were done at 0–4 °C. Cells were washed once in XB extraction buffer (40 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 10% glycerol and 1 mM PMSF), disrupted by glass beads in 10 ml XB buffer, and centrifuged at 40,000 r.p.m. for 1 h using a Beckman 50.2Ti rotor. The supernatant was bound batchwise to 1 ml of Ni-NTA agarose (Qiagen)¹⁶ for 2 h. The resin was washed sequentially with 10 ml XB containing 20 mM imidazole, then 10 ml buffer N (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10% glycerol, 0.02% NP-40 and 0.1 mM PMSF) containing 20 mM imidazole, and poured onto a column. After the resin was washed with 5 ml buffer N containing 20 mM imidazole, bound proteins were eluted sequentially with 5 ml buffer N containing 200 mM imidazole and then 5 ml buffer N containing 1 M imidazole. Fractions containing Cut3 and/or Cut14 were eluted by the addition of 200 mM imidazole and stored at –80 °C. Cut3 was partly cleaved during isolation procedures, but if the procedures were rapidly done in a small-scale preparation, the complex containing non-cleaved Cut3 could be obtained. This preparation also had the renaturation activity. The cellular content of Cut3 in non-overproducing wild type was determined to be 3×10^3 molecules per cell using recombinant Cut3 as standard for immunoblot, equivalent to one molecule per 8 kb genomic DNA.

DNA reannealing assay. Procedures similar to those described¹⁸ were used. Complementary DNA strands were obtained by heat-denaturing 3 kb long EcoRV-linearized pBluescript KS(+). Reaction mixtures (final volume 20 µl) contained the buffer A (20 mM Tris-HCl, pH 7.5, containing 10% glycerol, 50 mM NaCl, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT), 12 µM (as nucleotides) heat-denatured DNA and the protein fractions (the Cut3–Cut14 complex, 30–60 nM; RecA, 400 nM). For ATP-free reactions, buffer B (20 mM Tris-HCl at pH 7.5, containing 10% glycerol, 100 mM NaCl, 1 mM DTT) was used instead of buffer A. Reaction mixtures were incubated at 30 °C for various lengths of time. The reaction was terminated by adding 2 µl of 8% SDS and 100 mM EDTA. Samples were analysed on 0.7% agarose gel at 1.4 V cm^{–1} for 16 h, and DNA was visualized by ethidium bromide staining.

Sucrose density-gradient centrifugation. A linear sucrose gradient (5.0 ml, 10–40%) made in HB buffer¹⁷ was overlaid with 0.1 ml Cut3–Cut14 complex solution (4.5 µg ml^{–1}) or cell extracts (equivalent to 4×10^8 cells) and run at 40,000 r.p.m. for 16 h at 4 °C. We analysed 20 fractions by immunoblot using antibodies against Cut3 or Cut14HA.

Identification of mutation sites. Approximate location of the mutation sites was determined by integration rescue. Genomic DNAs were isolated from the corresponding regions of the temperature-sensitive mutant genome, followed by nucleotide sequence determination.

Preparation of nuclear chromatin and S1 nuclease digestion. The procedures previously described for nuclear chromatin²⁰ were followed. As added 530 U of S1 or 100 U of mung-bean nuclease (TaKaRa) to 70 µl chromatin of *S. pombe* cells, incubated at 36 °C for 30 min. The reaction was terminated by 50 mM EDTA. DNAs were extracted after SDS lysis.

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Metal ion catalysis during splicing of premessenger RNA

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The removal of intervening sequences from premessenger RNA is essential for the expression of most eukaryotic genes. The spliceosome ribonucleoprotein complex catalyses intron removal by two sequential phosphotransesterification reactions¹, but the catalytic mechanisms are unknown. It has been proposed that two divalent metal ions may mediate catalysis of both reaction steps, activating the 2'- or 3'-hydroxyl groups for nucleophilic attack and stabilizing the 3'-oxanion leaving groups by direct coordination². Here we show that in splicing reactions with a precursor RNA containing a 3'-sulphur substitution at the 5' splice site, interaction between metal ion and leaving group is essential for catalysis of the first reaction step. This establishes that the spliceosome is a metalloenzyme and demonstrates a direct parallel with the catalytic strategy used by the self-splicing group I intron from *Tetrahymena*³. In contrast, 3'-sulphur substitution at the 3' splice site provides no evidence for a metal ion–leaving group interaction in the second reaction step, suggesting that the two steps of splicing proceed by different catalytic mechanisms and therefore in distinct active sites.

Substitution of an oxygen leaving group by sulphur⁴ provides a means to test metal ion–leaving group interactions because various metals differ in their ability to coordinate sulphur: for example, Mn²⁺ readily accepts sulphur as a ligand, whereas Mg²⁺ does not^{5–8}. Thus, a switch in metal specificity from Mg²⁺ to Mn²⁺ following

sulphur substitution is evidence for a direct metal ion–leaving group interaction³.

We used enzymatic ligation⁹ to synthesize an adenovirus-derived splicing substrate (Ad5-1S) containing a single 2'-deoxy-3'-thio-uridine at the 5' splice site (Fig. 1a), in which sulphur replaces the 3'-oxygen leaving group in the first step of splicing. We also prepared the standard transcribed adenovirus substrate¹⁰, as well as ligated control substrates containing a single 2'-deoxyuridine (Ad5-1D) or unmodified uridine (Ad5-1R) at the 5' splice site. The absence of a 2'-hydroxyl group at the 5' splice site is known to have no effect on the rate or accuracy of pre-mRNA splicing⁹. All four substrates were tested for *in vitro* splicing in EDTA-pretreated HeLa nuclear extract (Fig. 2a). The control adeno transcript and the ligated substrates Ad5-1R and Ad5-1D all behaved similarly: no splicing occurred in the absence of exogenous divalent metal ions (Fig. 2a, lanes 2, 6 and 10), and efficient splicing occurred in the presence of 1.5 mM MgCl₂ (Fig. 2a, lanes 3, 7 and 11) or 1.5 mM MnCl₂ (Fig. 2a, lanes 4, 8 and 12). The Ad5-1S substrate also failed to undergo splicing in the absence of exogenous divalent metal ions (Fig. 2a, lane 14); however, in contrast to the control substrates, 1.5 mM MgCl₂ was unable to support splicing (Fig. 2a, lane 15). This inhibition of splicing in MgCl₂ was apparent at all times up to 2 hours, and at MgCl₂ concentrations up to 3.5 mM (data not shown). When the splicing buffer contained 1.5 mM MnCl₂, 5'-splice-site cleavage and lariat formation occurred efficiently (Fig. 2a, lane 16), but the second step of the reaction did not. Poor nucleophilicity of sulphur at phosphate centres¹¹, which has been observed in both non-enzymatic¹² and enzymatic¹³ reactions, may be the reason for inhibition of the second step of splicing. The appearance of splicing intermediates depended on added nuclear extract (Fig. 2a, lane 17) and therefore did not simply result from incubation of the 3'-thio-modified substrate under these buffer conditions. A 2'-O-methyl oligonucleotide that sequesters the 5' end of U1 small nuclear (sn) RNA¹⁴ blocked the reaction, but a nonspecific oligonucleotide did not, indicating that the spliceosome was responsible for 5'-splice-site cleavage of the modified substrate (data not shown).

To demonstrate the accuracy of the Ad5-1S splicing reaction in MnCl₂, we synthesized a fragment of Ad5-1S pre-mRNA with a single radiolabelled phosphate adjacent to the 2'-deoxy-3'-thio-uridine residue (Fig. 2b, top). The sulphur–phosphorus bond of a

Figure 1 Modified adenovirus pre-mRNA substrates for *in vitro* splicing. The pre-mRNAs are shown schematically at the top, with splice-site sequences underneath. The structures of the nucleosides flanking the 3'-S-phosphorothiolate bond are shown at the bottom. **a**, In the Ad5-1S substrate, the guanosine at 5'-splice-site position-1 was changed to 2'-deoxy-3'-thio-uridine, and the guanosine at position-2 to cytosine to avoid formation of a cryptic 5' splice site²⁵. **b**, In the Ad3-1S substrate, the guanosine at 3'-splice-site position-1 was changed to 3'-thioinosine.

Figure 2 A 3'-thio substitution at the 5' splice site results in a metal specificity switch. **a**, HeLa nuclear extract was used for *in vitro* splicing reactions with adenovirus transcript (lanes 1–4), Ad5-1R (lanes 5–8), Ad5-1D (lanes 9–12), or Ad5-1S (lanes 13–17) pre-mRNAs. Lanes 1, 5, 9 and 13 are unspliced RNAs. Splicing reactions contained no exogenous divalent metal ions (lanes 2, 6, 10 and 14), 1.5 mM MgCl₂ (lanes 3, 7, 11 and 15), or 1.5 mM MnCl₂ (lanes 4, 8, 12, 16 and 17). In lane 17, buffer replaced nuclear extract. Splicing precursor, intermediates and products are shown schematically on the right; the asterisk denotes residual unligated 3' half RNA. With the Ad5-1D substrate, a product of slightly decreased mobility relative to exon 1 intermediate is reproducibly observed when the reactions lack exogenous divalent metal ions (lane 10). This product is not accompanied by the appearance of any lariat species, and is therefore unlikely to be related to splicing. **b**, The spliceosome accurately cleaves the sulphur–phosphorus bond of Ad5-1S pre-mRNA in the presence of Mn²⁺. The strategy for mapping the 5' splice site is shown at the top (also see text). Standards are identified on the right. The upper band in lanes 2 and 3 (indicated by a dot) may be identical to the Cp*(dU)_{SH} dinucleotide, except that the sulphhydryl group became oxidized during work-up.

3'-S-phosphorothiolate linkage can be specifically hydrolysed in the presence of dilute aqueous AgNO_3 ; normal phosphodiester linkages are unaffected^{15,16}. Ag^+ treatment combined with RNase T₁ digestion produces the dinucleotide 5'-Cp*(dU)_{SH}-3', containing a free 3'-thiol (Fig. 2b, lane 2). RNase T₁ digestion of the purified exon 1 intermediate from an Ad5-1S/Mn²⁺ splicing reaction yielded the identical 5'-Cp*(dU)_{SH}-3' dinucleotide (Fig. 2b, lane 3); iodoacetamide, which reacts specifically with sulphhydryls, quantitatively derivatized the dinucleotide to the more slowly migrating acetamide thioether¹⁶, consistent with the presence of the 3'-thiol (data not shown). These results confirm that the spliceosome cleaved the sulphur-phosphorus bond of the Ad5-1S substrate.

The inhibition of Ad5-1S splicing in MgCl_2 could arise from a failure of the spliceosomal complex to assemble, rather than from a defect in the chemical step of the splicing reaction. We monitored the assembly of splicing complexes on the Ad5-1S and Ad5-1D substrates in the presence of MgCl_2 by electrophoresis in non-denaturing gels¹⁷ (Fig. 3). Assembly of the prespliceosomal A complex, the spliceosomal B complex and the catalytically activated C complex was equally efficient with Ad5-1S and Ad5-1D (Fig. 3), even though Ad5-1S did not give rise to splicing intermediates or products in the presence of MgCl_2 (Fig. 2a, lane 15). Thus the 3'-thio modification does not interfere with spliceosome assembly in the presence of MgCl_2 , but rather blocks some subsequent step, such as catalysis of phosphotransesterification within the assembled spliceosomal active site.

If the inhibition of Ad5-1S splicing in MgCl_2 is due to a specific defect in the chemical step of the reaction, rather than being a general consequence of having the modified linkage in the vicinity of the 5' splice site, then substitution of a nearby nucleoside with 2'-deoxy-3'-thiouridine should have no effect on splicing activity. We synthesized pre-mRNAs containing uridine, 2'-deoxyuridine, or 2'-deoxy-3'-thiouridine two bases downstream of the splice site (Ad5 + 2R, Ad5 + 2D and Ad5 + 2S, respectively) in the highly conserved GU dinucleotide. All three ligated pre-mRNAs were

spliced as efficiently as the transcribed control pre-mRNA (data not shown), indicating that the inhibition of splicing in MgCl_2 is specific to 3'-thio substitution at the 5' splice junction itself. Efficient splicing of the Ad5 + 2D and Ad5 + 2S substrates also demonstrates that spliceosome assembly and splicing do not require the 2'-hydroxyl group of the U residue within the nearly invariant GU dinucleotide, as inferred from oligonucleotide *trans*-splicing reactions¹⁸.

Given that Mn²⁺ coordinates sulphur much more effectively than does Mg²⁺ (refs 5–8), the strong preference for Mn²⁺ by the 5'-splice-site-substituted precursor implies that the sulphur atom interacts with Mn²⁺ during the catalytic process. We infer that there could be an analogous metal ion-oxygen interaction during splicing of the unmodified precursor (Fig. 4). Before reaction, this interaction is expected to be weak or even repulsive¹⁹. As the scissile phosphodiester bond breaks, the 3'-oxygen develops negative charge in the transition state and the interaction with the metal ion should strengthen. An appropriately positioned metal ion in the spliceosomal active site could therefore selectively stabilize the transition state relative to the ground state, enhancing the reaction rate. Although it is not known whether protein or RNA mediates the phosphotransesterification reactions in the spliceosome, we have shown that the first step of pre-mRNA splicing occurs by a strategy that is amenable to catalysis by RNA³.

2'-Deoxy-3'-thiouridine could not be used as a probe for metal ion catalysis at the 3' splice site for two reasons: an absence of the 2'-hydroxyl group at the 3' splice site causes a 95% decrease in the efficiency of exon ligation⁹; and mutation of the highly conserved guanosine at the intron terminus to uridine abolishes pre-mRNA splicing²⁰. However, site-specific substitution of inosine for guanosine at the 3' splice site does not impair the rate or accuracy of pre-mRNA splicing *in vitro*²¹. Because an RNA dinucleotide containing 3'-thioinosine had already been characterized¹⁶, we developed a synthetic route to the phosphoramidite of 3'-thioinosine rather than 3'-thioguanosine (S.S. and J.A.P., manuscript in preparation).

We synthesized an adenovirus-derived splicing substrate (Ad3-1S) containing a single 3'-thioinosine at the 3' splice site (Fig. 1b), in which sulphur replaces the 3'-oxygen leaving group in the second step of splicing. We also prepared the standard transcribed adenovirus substrate¹⁰, as well as ligated control substrates containing a

Figure 3 The 3'-thio modification does not inhibit spliceosome assembly when Mg^{2+} is present as the sole divalent metal ion. The Ad5-1D (lanes 1–6) or Ad5-1S (lanes 7–12) pre-mRNAs were spliced *in vitro* for the times indicated at the top of each lane, and splicing complexes were fractionated by electrophoresis on a non-denaturing 4% polyacrylamide gel¹⁷. The mobilities and kinetics of non-specific H complex, pre-spliceosomal A complex, spliceosomal B complex and catalytically activated spliceosomal C complex (indicated on the right) conform to previously observed patterns.¹⁷

Figure 4 A catalytic metal ion is present in the spliceosomal active site for the first step of pre-mRNA splicing. The three nucleotides that participate directly in the first step of pre-mRNA splicing—the last base of exon 1, the branch point adenosine, and the first base of the intron—are shown bound to sites 1, 2 and 3 within the spliceosomal active site². A catalytic magnesium ion is inferred from this work to bind in the spliceosomal active site and directly coordinate the 3'-oxyanion leaving group in the proposed transition state. Adapted from ref. 2.

guanosine (Ad3-1G) or inosine (Ad3-1I) at the 3' splice site, and tested all four substrates for *in vitro* splicing in EDTA-pretreated HeLa nuclear extract (Fig. 5). No splicing occurred in the absence of exogenous divalent metal ions (Fig. 5, lanes 2, 6, 10 and 14). As expected, efficient splicing occurred with the control adeno transcript and the Ad3-1G and Ad3-1I ligated substrates in the presence of either 1.5 mM $MgCl_2$ (Fig. 5, lanes 3, 7 and 11) or 1.5 mM $MnCl_2$ (Fig. 5, lanes 4, 8 and 12). In contrast to the results obtained at the 5' splice site, however, both steps of splicing occurred efficiently with the Ad3-1S 3'-thio substituted pre-mRNA in the presence of either of the divalent metal ions Mg^{2+} or Mn^{2+} (Fig. 5, lanes 15 and 16). This result was obtained several times using different nuclear extracts and different metal ion preparations (data not shown). Treatment of Ad3-1S pre-mRNA with $AgNO_3$ (ref. 15) gave nearly quantitative conversion to the expected hydrolysis products (data not shown), confirming the presence of the 3'-S-phosphorothiolate linkage in the pre-mRNA. Sequencing of the Ad3-1 spliced products from splicing reactions carried out in either Mg^{2+} or Mn^{2+} showed that the correct 3' splice site was used in both cases (data not shown). The possibility that added Mg^{2+} displaces trace protein-bound thiophilic metal ions, freeing them to support splicing of the 3'-thio-substituted pre-mRNA, is highly unlikely; our splicing reactions contained 0.8 mM EDTA, and the EDTA stability constants of most thiophilic divalent metal ions are at least five orders of magnitude greater than that of Mg^{2+} (ref. 22). Hence, almost all released thiophilic metal ions would have been chelated by EDTA. Furthermore, such a displacement effect might have resulted in splicing of the 5'-splice-site-substituted pre-mRNA in Mg^{2+} , which we did not observe (Fig. 2a, lane 15). We conclude that the sulphur leaving group in the exon ligation reaction does not require the presence of a metal ion to which it can coordinate effectively.

A switch in metal specificity could be masked because the chemical step of exon ligation may not be rate-limiting; however, it seems likely that disruption of an important metal ion interaction would have reduced the efficiency of splicing. For example, in the case of the Klenow fragment, there is structural evidence for a metal

ion-leaving group interaction at the 3'-5' exonuclease active site²³, and thio substitution of the leaving group decreases k_{cat}/K_M by ~50,000-fold in the presence of Mg^{2+} (J. F. Curley, C. M. Joyce, and J.A.P., manuscript submitted). Alternatively, there may be no interaction between a metal ion and the leaving group in the second chemical step of splicing. The spliceosome may stabilize the leaving group by some means other than that observed in the first reaction step, such as general acid catalysis and/or hydrogen-bond donation: the source of this proton or hydrogen-bond donor could be a metal-bound water molecule, an RNA residue with a locally perturbed pK_a , or a spliceosomal protein side chain. The first and second reaction steps probably occur at distinct active sites: for example, blockage of both reaction steps by the same phosphorothioate diastereomer argues that they are not simply forward and reverse reactions at a single active site²⁴, where the same metal ion stabilizes the leaving group in the first step and activates the nucleophile in the second step². Our observation that 3'-thio substitution blocks Mg^{2+} -mediated splicing at the 5' splice site but not the 3' splice site argues against the alternative possibility that the two steps proceed as parallel reactions in a single active site, in which the same metal ion stabilizes the leaving group in both steps. □

Methods

Pre-mRNA substrates. We synthesized 5'-O-monomethoxytrityl-2'-deoxy-3'-thiouridine 3'-S-phosphoramidite as described³; synthesis of the 5'-O-dimethoxytrityl-2'-O-t-butyl dimethylsilyl-3'-thioinosine-3'-S-phosphoramidite will be reported elsewhere. All dinucleotides and oligonucleotides were synthesized on a Millipore solid-phase DNA/RNA synthesizer and deprotected and purified using standard procedures, except that 2'-deoxy-3'-thiouridine and 3'-thioinosine were incorporated as described⁴. We synthesized the following dinucleotides and oligonucleotides: 5'-(dU)₆pG-3', 5'-(dU)pG-3', Ad5-1R/21 (5'-UGUGA-GUACUCCUCUCAAAA-3'), Ad5-1D/21 (5'-(dU)GUGAG UACUCCUCUCAAAA-3'), Ad5-1S/21 (5'-(dU)₆GUGAGUACUCCUCUCAAAA-3'), Ad5-2-10p (5'-pCAGCUGUUGC-3'), Ad3-1G (5'-AGCUCG CGGUU-3'), Ad3-1I (5'-AICUCGCGGUU-3'), and Ad3-1S (5'-AI₃CUC GCGGUU-3') ((dU) is 2'-deoxyuridine, (dU)₆ is 2'-deoxy-3'-thiouridine, p is phosphate, I is inosine, and I₃ is 3'-thioinosine).

For the experiments shown in Figs 2a and 3, we synthesized the Ad5-1 substrates as described²⁵, except that the dinucleotides UpG (Sigma), (dU)pG, or (dU)₆pG were used to initiate transcription of the 3' half RNA, and the 3' half RNA was labelled internally with [α -³²P]UTP. We synthesized the Ad5 + 2 substrates as described²⁵, using the dinucleotides UpG, (dU)pG or (dU)₆pG to initiate transcription of the 3' half RNA. For the experiment shown in Fig. 2b, we synthesized the Ad5-1 substrates with a single ³²P-phosphate adjacent to the modified nucleotide. The Ad5-1R/21, Ad5-1D/21 and Ad5-1S/21 oligonucleotides were 5'-³²P-phosphorylated and ligated²⁶ to the Ad5-2-10p oligonucleotide to generate the fragments Ad5-1R/30, Ad5-1D/30 and Ad5-1S/30, respectively. We then ligated these fragments to Ad5-10 5' RNA (corresponding to the first 57 nucleotides of exon 1) and Ad5 + 21 3' RNA (corresponding to the last 223 nucleotides of the intron and all 99 nucleotides of exon 2).

For synthesis of the Ad3-1 substrates, the Ad3-1G, Ad3-1I and Ad3-1S oligonucleotides were 5'-³²P-phosphorylated and ligated²⁶ to the Ad3-3 5' RNA (corresponding to exon 1 and the first 241 nucleotides of the intron) and the Ad3 + 10 3' RNA (corresponding to nucleotides 10–99 of exon 2). Before the ligation reactions, we 3'-end-labelled the Ad3 + 10 3' RNA with [α -³²P]cordycepin triphosphate using yeast poly(A) polymerase (US Biochemicals).

Nuclear extracts and *in vitro* splicing reactions. HeLa cells were purchased from the National Cell Culture Center (Minnesota), and nuclear extract prepared as described²⁷, except that dialysis buffer contained 42 mM $(NH_4)_2SO_4$ instead of KCl. For *in vitro* splicing, nuclear extract (already containing 0.2 mM EDTA) was supplemented with an additional 2.5 mM EDTA²⁸, and incubated on ice for 30 min. Splicing reactions (10 μ l) included 30% nuclear extract (final EDTA concentration 0.8 mM), 0.5 mM ATP, 0.1 mM cordycepin triphosphate, 20 mM creatine phosphate, 20 ng μ l⁻¹ carrier RNA, trace quantities of radiolabelled splicing substrate, and 1–2 units μ l⁻¹ RNA-guard RNase inhibitor (Promega). We also included water, $MgCl_2$ or $MnCl_2$, as described in the text and figure legends; metal ion chlorides (Aldrich) were >99.99%

Figure 5 Mg^{2+} supports *in vitro* splicing of a pre-mRNA with a 3'-thio substitution at the 3' splice site. *In vitro* splicing reactions were carried out with adenovirus transcript¹⁰ (lanes 1–4) and Ad3-1G (lanes 5–8), Ad3-1I (lanes 9–12), and Ad3-1S (lanes 13–17) ligated RNAs as for Fig. 2a. Splicing precursor, intermediates and products are shown schematically on the right. Stars indicate background cleavage products (RNA containing an inosine 3'-S-phosphorothiolate linkage cleaves ~2,000-fold faster than unmodified phosphate-linked RNA¹⁶). For the ligated pre-mRNAs, the exon 1 intermediate contains no radiolabel and is therefore not visible.

pure. Splicing reactions were for 1–2 h at 30 °C. Extracted and precipitated RNAs were fractionated on a denaturing 8% polyacrylamide gel; the gel and electrophoresis buffer (1 × TBE) contained 10 mM dithiothreitol (DTT) and gels were pre-run for at least 6 h.

Splice-site mapping. For 5′-splice-site mapping, we included the Ad5-1S pre-mRNA in splicing reactions as described and gel-purified the 5′ exon intermediate. To generate a standard, the Ad5-1S/30 fragment was treated with AgNO₃ (ref. 15), and the 10-nucleotide labelled product of silver cleavage (5′-pCAGCUGUUGC(dU)_{SH}-3′) was purified in a 20% polyacrylamide denaturing gel containing 10 mM DTT. Following elution, we desalted the oligonucleotide in a Sep-Pac C₁₈ cartridge (Waters) and dried it *in vacuo*. The purified 5′ exon intermediate, the Ag⁺-cleaved fragment, and the Ad5-1 pre-mRNA were digested with RNase T₁ and fractionated in a 20% polyacrylamide denaturing gel containing 10 mM DTT. RNase T₁ digestions⁹ contained 10 mM DTT.

For 3′-splice-site mapping, extracted and precipitated RNAs from 100-μl splicing reactions (Fig. 3b, lanes 15 and 16) were reverse transcribed using a DNA primer (Ad.33) complementary to the 3′-terminal 21 nucleotides of the Ad3-1S substrate. We used the products of these reactions as PCR templates, with Ad.33 primer and another oligonucleotide (Ad.55) corresponding to the 5′-terminal 20 nucleotides of the Ad3-1S substrate, and sequenced the PCR products derived from ligated exons.

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A second catalytic metal ion in a group I ribozyme

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Although only a subset of protein enzymes depend on the presence of a metal ion for their catalytic function, all naturally occurring RNA enzymes require metal ions to stabilize their structure and for catalytic competence¹. In the self-splicing group I intron from *Tetrahymena thermophila*², several divalent metals can serve structural roles, but only Mg²⁺ and Mn²⁺ promote splice-site cleavage and exon ligation^{3,4}. A study of a ribozyme reaction analogous to 5′-splice-site cleavage by guanosine uncovered the first metal ion with a definitive role in catalysis. Substitution of the 3′-oxygen of the leaving group with sulphur resulted in a metal-specificity switch, indicating an interaction between the leaving group and the metal ion⁵. Here we use 3′-(thioinosyl)-(3′ → 5′)-uridine⁶, IspU, as a substrate in a reaction that emulates exon ligation. Activity requires the addition of a thiophilic metal ion (Cd²⁺ or Mn²⁺), providing evidence for stabilization of the leaving group by a metal ion in that step of splicing. Based on the principle of microscopic reversibility, this metal ion activates the nucleophilic 3′-hydroxyl of guanosine in the first step of splicing, supporting the model of a two-metal-ion active site⁷.

The self-splicing reaction performed by the *Tetrahymena* intron consists of two consecutive phosphotransesterification reactions. The mechanism of the first step, 5′-splice-site cleavage, has been studied using a truncated form of the intron (L-21 ScaI) in the forward reaction (G + CCCUCUpA → GpA + CCCUCU, where only the reactive phosphate is shown explicitly) (Fig. 1a)^{2,8}. The second step of splicing, exon ligation, is chemically the reverse of the first step⁹ and is thought to occur in the same active site^{10,11}. This step is mimicked by the reverse of the ribozyme reaction (GpN + CCCUCU → G + CCCUCUpN, where N is A, C or U) (Fig. 1a)^{12–14}. Kinetic studies of this reverse reaction have used dinucleotides with guanosine at the 5′-position. However, inosine will substitute for guanosine in both splicing and the enzymatic forward reaction^{15,16}. For ease in chemical synthesis, we chose IspU, an inosine-containing dinucleotide with a 3′-S-phosphorothiolate linkage⁶, to prospect for a metal ion interacting with the leaving group in the reverse reaction (Fig. 1b).

Under conditions previously shown to support the reverse reaction efficiently, namely pH 7.0 and high MgCl₂ concentration (110 mM)¹³, only the unmodified dinucleotide, IpU, was a substrate for transesterification (Fig. 2). Addition of Mn²⁺ (1–100 mM) resulted in progressively higher rates of transesterification of IspU (Fig. 2). To find conditions under which the observed rate constant (*k*_{obs}) reflects the chemical step, a pH profile was determined for the