might only be able to detect genes capable of being expressed in fibroblasts. Thus the value of the 3T3 assay to detect the genes involved in most human malignancies may be questionable.

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22. We thank G. Vuocolo and S. Goldflam for technical assistance and K. Pinette for preparation of the manuscript. J.E. is a Monica Shander Fellow of the Wistar Institute and is supported by training grant CA 09171. Supported by NIH grant GM 31060 (K.N) and NCI grants CA 10815, CA 25878, and CA 16685 (C.M.C.).

7 March 1984; accepted 13 March 1984

Molecular Model for Messenger RNA Splicing

Abstract. *A molecular model is presented for a messenger RNA (mRNA) "splice region." The model requires cation coordination to reduce backbone-backbone electrostatic repulsion and it allows for every base residue on the pre-mRNA to be stacked in A-form helical geometry with a recognition element on the intron or exon (or both) sides of the splice junction. The two nucleotides involved in the initial steps of the cleavage-ligation mechanism must adopt a non–A-form geometry, which ideally positions reactive groups on the pre-mRNA for the necessary catalytic chemistry. The model is also consistent with available biochemical data on splicing reactions.*

RNA splicing is now established as a major RNA processing reaction in eukaryotic cells (*1*). Proposals for the selection of the 3′ and 5′ splice sites and the removal of intron sequences have been made by Murray and Holliday (*2*), Lerner *et al.* (*3*), Rogers and Wall (*4*), as well as others (*5, 6*). These proposals are schematic and thus do not provide for the assessment of their stereochemical, thermodynamic, and mechanistic plausibility. We now describe a molecular model of a messenger RNA (mRNA) "splice region" that not only satisfactorily accounts for the stereochemical and mechanistic features of cleavage and ligation, but is thermodynamically reasonable and consistent with biochemical data on splicing reactions.

The splice region structure shown in Fig. 1 is consistent with mRNA splicing complexes discussed previously (*2–6*). Although independently derived, it is the structural counterpart of a previously proposed B-DNA crossed strand–exchange molecular model for genetic recombination (*7*). It is composed of an mRNA transcript which, reading 5′ to 3′, enters at arrow A as the 5′ exon, leaves at arrow B as the intron, enters again at arrow C, and finally leaves at arrow D as the 3′ exon. The other strands represent recognition elements, shown in Fig. 1 as splicer RNA's (SR), which serve to juxtapose the 3′ and 5′ splice sites by bonding specifically to consensus bases on either the intron or exon sides.

A compilation of mRNA splice site sequences shows that there are many which are compatible with splicing, although extensive consensus sequences have been derived for both the 5′ site,

$$(^{C}_{A})\text{-A-G//G-T-}(^{A}_{G})\text{-A-G-T}$$

and the 3′ site,

$$(^{T}_{C})_{n}\text{-N-}(^{C}_{T})\text{-A-G//G}$$

(A, adenine; C, cytosine; G, guanine; T, thymine; N, any base) (*3, 4, 8*). The important but limited role of base sequence at the 5′ site has been demonstrated by the observation that a sequence change from GT to AT (*9*) or GT to GG (*10*) abolishes splicing, but any single transition mutation at one of the other purine sites does not detectably affect splicing (*9*). It should be appreciated that the model in Fig. 1 is not dependent on the exact base sequence shown, but instead requires that there be sufficient complementarity between whatever 5′ and 3′ splice site sequences are present and the available recognition elements.

As suggested by a number of authors (*3–5*), good candidates for the recognition elements are small nuclear RNA's (snRNA). Perhaps the most likely snRNA involved in mRNA splicing is U1 RNA for a number of reasons (*11*). The 5′ terminus of the U1 RNA is highly complementary to consensus sequences on the intron side of both the 5′ and 3′ splice sites. Also, although whole HeLa nuclei were used, antibodies to Sm or ribonucleoproteins, which can precipitate U1 ribonucleoproteins, have been shown to inhibit adenovirus splicing (*12*). Finally, it has been shown that U1 RNA selectively binds to the 5′ splice site of β-globin mRNA (*13*). Also, U2 RNA is complementary with the consensus splice sequences on the exon side. Both U1 and U2 may aid in the alignment of splice sites (*5*). To demonstrate that splicer RNA's can be positioned on both the intron and exon sides (as well as ease the construction of the helical regions), two splicer RNA's have been incorporated into the molecular model seen in Fig. 1.

It is well known that helical RNA prefers the A-form (*14*) in part because of the intramolecular water bridges that form between adjacent ribose 2′-hydroxyl and 3′-phosphate groups (*15*) and the sterically unfavorable interactions of the 2′-hydroxyl group that would arise in helical forms other than A (*16*). The two double-helical regions of the molecular model shown in Fig. 1 (L and R) are in the standard A-form, although one (but probably not both) of these helical regions could be composed of the A-form mRNA transcript alone with no RNA recognition element (SR) as proposed earlier (*3–6*). Whereas amino acid residues and nucleic acid bases can undergo hydrogen-bonding with some degree of specificity (*17*), it is possible that splicing proteins can act in concert with SR's to align the splice sites. There is considerable availability of hydrogen bonding sites in the grooves of the helices (Fig. 1), and this specificity can be fostered by the ability of protein main-chain atoms and nucleic acid backbone atoms to adopt compatible structures (*18*).

Although the helical regions are in A-form, the splice region (boxed in Fig. 1) must assume a different geometry. This non–A-form region is made up of the two sugar moieties at the 3′ end of the first

(5′) exon and the 3′ intron end and each of the five adjacent backbone dihedral angles reading 5′ to 3′ (Table 1). [These changes require the concomitant loss of the intramolecular water bridge between the 2′-hydroxyl and 3′-phosphate groups in the non–A-form region. But since these water molecules must be stripped for the mechanism to proceed (below), formation of a non–A-form splice region must not be viewed as unreasonable.] A variety of positions were explored for the intron and exon strands, but no others were found that could achieve a mechanistically plausible geometry, while maintaining the A-form throughout L and R and avoiding sterically forbidden interactions between the helical regions.

Of the ten dihedral angles in the splice region model (Table 1), none are within 10 degrees of the corresponding angle in a regular A-RNA duplex (*14*), and only two are within 40 degrees. One might therefore suppose that such dihedral angles are unfavorable for an RNA structure. However, when these ten splice region angles are compared to the closest corresponding angles in yeast phenylalanine transfer RNA ($tRNA^{Phe}$) (*19*), seven of the ten splice region angles are within 10 degrees and two more are within 41 degrees of those in the tRNA. The only angle of the ten which does not compare well with a corresponding $tRNA^{Phe}$ value is relatively close in value to a corresponding angle in the DNA crossed strand–exchange model (*7*). Since the dihedral angles of the splice region are not very different from observed domains, our model is a conformationally reasonable transition state configuration. Moreover, it has been observed that four hexadecadeoxynucleotides can form a stable tetrameric junction complex in solution (*20*). Unfortunately, similar results are not yet available for the ribose case, although there is no apparent reason why they should not act similarly (*21*).

In the splice region, there are ten oxygen atoms in close proximity (Fig. 2), creating an unfavorable distribution of negative charge. Although the phosphorus atoms afford some counterbalance, presumably a strong electrophile such as a magnesium ion or active site cationic amino acid residue (or both) is also required to coordinate with the oxygens to reduce the electrostatic repulsions. The model is uncrowded around the splice region (Fig. 1) and thus presumably would not sterically prevent such necessary metal cation or protein residue coordination. In addition, a cation (or cations) probably is required to play a mechanistic role (see below).

Table 1. Splice region backbone dihedral angles (degrees).

Backbone dihedral angle	Splice region model: 5′ Exon to intron	Splice region model: Intron to 3′ exon	A-RNA regular duplex*	Closest corresponding angles† in yeast $tRNA^{Phe}$: 5′ Exon to intron	Closest corresponding angles† in yeast $tRNA^{Phe}$: Intron to 3′ exon	DNA crossed-strand–exchange model‡
C4′-C3′-O3′-P	180	135	−151	−175(C13)	174(U47)	−90,−100
C3′-O3′-P-O5′	105	−175	−74	100(m^5C49)	−172(D16)	150,150
O3′-P-O5′-C5′	10	60	−62	41(D17)	58(m^7G46)	80,150
P-O5′-C5′-C4′	30	−160	180	128(A36)	−159(D17)	180,80
O5′-C5′-C4′-C3′	−170	100	48	−174(Y37)	99(C13)	−60,−150

*From (*14*). †From (*31*). The tRNA nucleotide is given in parentheses. Abbreviations: C, cytidine; m^5 C, 5 methyl cytidine; D, dihydrouridine; A, adenosine; Y, y nucleoside; U, uridine; m^7 G, 7 methyl guanosine. ‡From (*7*). Angles for two symmetric models are given in original reference and are shown here.

Although energy calculations are not meaningful because of the lack of positional data of cation (or cations) and their consequence on neutralization and charge transfer in the splice region, we believe that the overall structure shown in Fig. 1 is sufficiently stabilized in the helical regions (that is, every base is fully stacked) to allow the high energy, mechanistically necessary splice region to exist transiently in a complex with cations and enzyme under physiological conditions.

There is direct evidence of binding of U1 RNA to the 5′ splice site of β-globin pre-mRNA; 3′ splice site binding is not observed (*13*). This is puzzling since the two exons must be juxtaposed for splicing (*22*). The splice model in Fig. 1 suggests that binding of a recognition element to both splice sites is stereochemically plausible. However, even with complete stacking and sufficient cations to reduce backbone-backbone electrostatic repulsion, the proposed splice site structure is probably only short-lived in solution because of its greatly reduced overall entropy and relatively limited 3′ splice site consensus sequence complementarity. Since the study of Mount *et al.* (*13*) could only detect U1 RNA binding to pre-mRNA when the half-life of the complex was about 10 minutes or more, these two results are not incompatible.

Upon formation of the model structure, each of the 2′-hydroxyl groups is remarkably placed in an ideal location for S_N2 attack on the adjacent 3′-phosphate. When a similar structure is built with B-form helices, or if the strands are placed in any other relative position

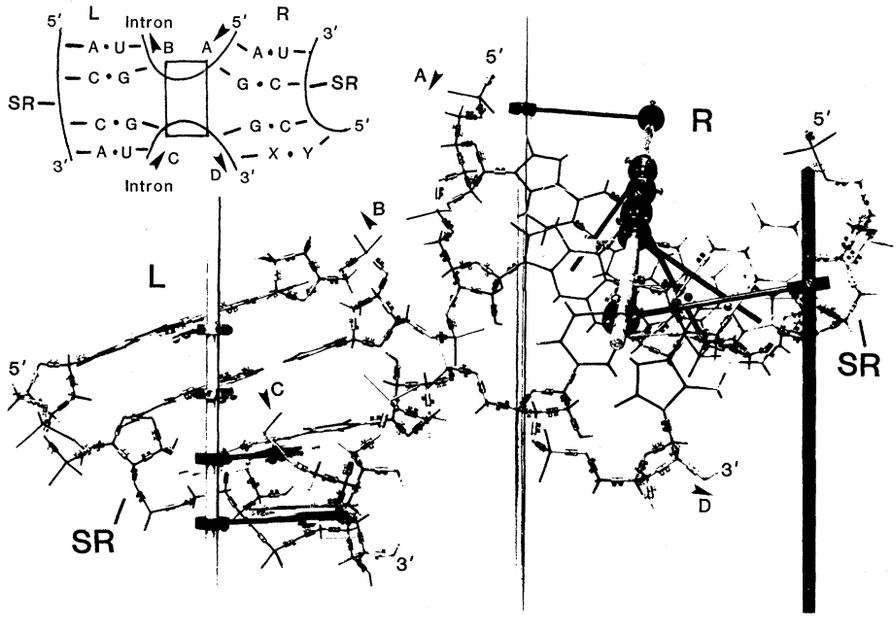

Fig. 1. Molecular model of mRNA splice region. Arrows A, B, C, and D show the movement of the mRNA through the model. The two fully stacked duplex helical regions are labeled L and R. The two complementary splicer RNA (SR) strands (recognition elements) are labeled SR. The boxed region in the schematic illustration indicates the splice region (see text and Fig. 2). The bases shown in the schematic are the consensus splice sites sequences. The 3′ end of the splicer RNA of the R helix is hidden in the wire model photograph.

while maintaining the A-form, it is in no way possible to position both hydroxyl groups similarly for such an attack.

The position of the reactive groups in the splice region, considered with a knowledge of available data on RNA splicing, suggests a general mechanism for mRNA splicing (Fig. 2). Most available biochemical evidence on the mechanism of RNA splicing has been obtained from tRNA splicing; in vitro systems with mRNA have only recently been developed (*23*). Since such systems have yet to yield details on the mechanism of mRNA splicing, we have made the assumption that general biochemical features of tRNA splicing apply to mRNA splicing. It should be appreciated that the mechanism and general structure of this molecular model might also apply to rRNA (ribosomal RNA) or tRNA splicing, although it is clear that there are significant differences between the splicing systems. For example, no splicer RNA has been identified in yeast tRNA splicing (*24*), and no *trans* acting RNA or even protein is required in *Tetrahymena* rRNA splicing (*25*).

The mechanism can be viewed as occurring in two steps. The first follows along the same lines as that established for ribonuclease A (*26*); it is essentially two "directly coupled" (*27*) ribonuclease A reactions. In this step, an endonuclease abstracts hydrogens from the 2′ OH groups (by general acid-base catalysis), and the anionic 2′ O atoms then attack the adjacent 3′-phosphates so that the bonds from 3′ P to 5′ O are cleaved and the electrons taken up by the 5′ O's. Thus, two 2′,3′-cyclic phosphates are formed. Experimental evidence from tRNA in yeast (*24*), *Chlamydomonas* (*28*), *Xenopus* oocytes (*29*), and a HeLa cell extract (*30*) suggest that a cyclic 2′,3′-phosphate terminus may be a universal intermediate structure in splicing. In yeast, it has been shown that the cyclic phosphate exists on both the 3′ end of the intron and on the 3′ end of the 5′ exon half-molecule after endonuclease cleavage (*24*). In addition, in vitro analysis of Pb(II)-catalyzed cleavage of yeast $tRNA^{Phe}$ backbone also has revealed that a cyclic 2′,3′-phosphate is formed (*31*). A divalent cation is not required for ribonuclease A, but it is probably necessary for the first step in the splicing mechanism, as mentioned, because of the energetic instability of the closely situated phosphate groups. Cation coordination can also accelerate S_N2 displacement in several ways, such as electron withdrawal from the leaving group, charge neutralization, induction of strain in the ground state, and stabilization of the transition state (*32*).

Either of two pathways, 2a or 2b, can then be followed in the second step. Phosphorylation of the 5′ OH group has been identified in tRNA splicing in yeast (*24*), and *Chlamydomonas* (*28*); in both organisms a mechanism similar to the first pathway proposed here was suggested to explain the existence of the 2′-phosphomonoester–3′,5′-phosphodiester splicing product. In pathway 2a, the exon 5′ OH would first be phosphorylated by a specific kinase. Subsequently, an oxygen of the 5′-phosphate would attack the cyclic 2′,3′-phosphate group breaking the 3′-oxygen-phosphate bond. The 3′ O anion can then attack the 5′-phosphate resulting in 2′-phosphomonoester–3′,5′-phosphodiester formation. In pathway 2b, a ligase would abstract the hydrogen from the exon 5′ OH, the exon 5′ O anion would then attack the exon cyclic 2′,3′-phosphate group directly (the exon 5′ O anion would already be ideally positioned to do so). As in the mechanism proposed for yeast and *Chlamydomonas* tRNA splicing (*24, 28*), adenosine triphosphate (ATP) would be used in pathway 2a as a phosphate source for a specific kinase to phosphorylate the 5′ hydroxyl group. In pathway 2b, and perhaps also in 2a, ATP could provide an energy source for a protein–nucleic acid conformational change, moving the 5′-oxygen to a position where attack and ligation must occur. In either pathway, once splicing is complete, the phosphate between the ligated exon strands would flip to yield a single-stranded transcript of A-form RNA. In pathway 2a, an additional phosphate would remain on the 2′-oxygen of the exon sugar and must be removed. Although the data are as yet far from conclusive, the finding that the phosphate bond after splicing is not associated with a 2′-phosphate group points to pathway 2b as the one used in mRNA splicing (*23*).

In vitro splicing systems (*23*) should reveal the biochemical requirements for mRNA splicing (whether there is a need for a splicer RNA recognition element) and the mechanistic details of the process (whether a cyclic 2′,3′-phosphate group is an intermediate, and, if so, whether pathway 2a or 2b is followed). Extension of the tetrameric junction structure studies of Kallenbach, Ma, and Seeman (*20*) to the ribose case can confirm that the RNA backbone possesses the inherent flexibility to form the junction presumed by the model. Incubation of such a structure in buffer with the

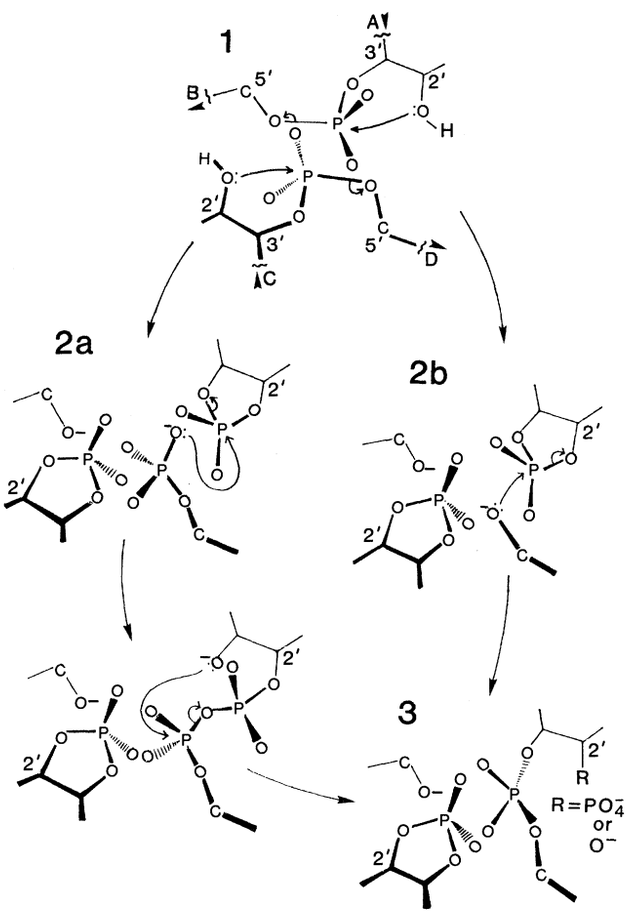

Fig. 2. Reaction mechanism for mRNA splicing as described in text. The reacting groups shown are located within the boxed region of Fig. 1 and their directionality is indicated by the arrows A, B, C, and D.

appropriate divalent cation could result in "spliced" RNA products being formed (*31*). These and other tests can help to evaluate the validity of the proposed model.

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21 November 1983; accepted 15 February 1984

Inhibition of Dihydropteridine Reductase by Novel 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Analogs

Abstract. *Hydroxylated derivatives of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a nigrostriatal neurotoxin in humans and primates, noncompetitively inhibited dihydropteridine reductase from human liver and rat striatal synaptosomes in vitro at micromolar concentrations. In contrast, MPTP and its chloro- and* nor*-derivatives did not inhibit this enzyme at lower than millimolar concentrations. Dihydropteridine reductase converts dihydrobiopterin to tetrahydrobiopterin, the required cofactor for the hydroxylation of aromatic amino acids during the synthesis of dopamine and serotonin.*

During the summer of 1982, several individuals with a history of drug addiction intravenously injected a "synthetic heroin" that was obtained from an illegal laboratory (*1*). The drug preparation contained 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) (0.3 percent), a meperidine analog that has analgesic properties, and the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (3.2 percent) that was formed presumably as a byproduct during the synthesis of the meperidine analog. Four of these individuals developed persistent parkinsonian symptoms and were studied extensively (*1*).

According to another report (*2*), one individual developed parkinsonism after using MPPP (and presumably MPTP) that he had synthesized. This person died of a drug overdose 2 years after the onset of his parkinsonism, and histological examination of his brain revealed marked destruction of the cells in the substantia nigra. In comparison, idiopathic parkinsonism in humans is characterized by a similar degeneration of the nigrostriatal dopaminergic pathway with specific loss of neurons in the substantia nigra accompanied by a marked decrease in the concentration of dopamine and its major metabolite, homovanillic acid, in the caudate nucleus and putamen (*3*). Administration of MPTP to rhesus monkeys has been shown to produce pathological and neurochemical changes that resemble closely idiopathic parkinsonism in humans (*4*), but MPTP does not produce neurotoxicity in the nigrostriatal dopaminergic system in guinea pigs or rats (*5*).

The metabolism and mode of action of MPTP has not been investigated systematically in man or other animals. What is known of the catabolism of xenobiotics by microsomal enzymes (*6*) suggests that MPTP could first be metabolized, either by aromatic hydroxylation or by aromatization of the tetrahydropyridine moiety (*7*) or both, to yield compounds that could react subsequently with one or more components of the nigrostriatal system. Studies in our laboratories have revealed that catechol- or hydroxy-containing aromatic compounds are potent, noncompetitive inhibitors (*8–10*) of DHPR [dihydropteridine oxidoreductase (NADH); E.C. 1.6.99.10]. This enzyme catalyzes the conversion of dihydrobiopterin to tetrahydrobiopterin, the required cofactor for hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine in the rate-limiting step of dopamine synthesis. We tested MPTP and nine of its analogs against DHPR in vitro to determine whether or not they inhibit this enzyme.

The 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) compound and its 4′-chlorophenyl analog were obtained as hydrochloride salts (Aldrich Chemical), and MPTP was obtained as a free base and converted to its hydrochloride salt. The 4′-chlorophenyl derivative of MPTP was prepared via the *N*-methylcarbamate, which was then reduced with lithium aluminum hydride. The various hydroxy-substituted analogs were obtained as described (*11*). All new compounds gave correct combustion analyses, and their structures were confirmed with spectral data.

Enzyme preparations were obtained from human liver purified by ammonium sulfate precipitation and sequential chromatography (on DEAE-Sephacel, Matrex Gel Blue A, and hydroxyapatite) (*8, 12*) and from prepared rat striatal synaptosomes (*13*). Each compound was tested for its ability to inhibit DHPR. After preincubation of each inhibitor with DHPR for 10 minutes at 25°C, the reaction rate of DHPR was determined (*14*)