Volume 13 Number 21 1985 Nucleic Acids Research

Self-catalyzed cyclization of the intervening sequence RNA of *Tetrahymena:* inhibition by intercalating dyes

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Received 27 May 1985; Revised and Accepted 4 October 1985

ABSTRACT

The intervening sequence (IVS) excised from the pre-rRNA of *Tetrahymena* undergoes a self-catalyzed cleavage-ligation reaction to form a covalently closed circular RNA. This cyclization reaction is kinetically inhibited by ethidium bromide (50% inhibition at 22 ± 14 μM, greater than 99% inhibition at 53 ± 16 μM for a 20 minute reaction). The dye does not alter the sites of the cyclization reaction, but it does increase the relative amount of reaction at a minor site 19 nucleotides from the 5' end of the IVS. The reversibility of the inhibition and the relative inhibitory strength of acridine orange, ethidium and proflavine suggest that inhibition is due to intercalation of the dye in functionally important secondary or tertiary structures of the IVS. The concentration of dye required to inhibit cyclization is much higher than expected from the known binding constants of such dyes to tRNA. At high Mg^{2+} to Na^{+} ratios, conditions which should stabilize RNA structure, a subpopulation of the IVS RNA molecules is resistant to ethidium inhibition, even at 200 μM ethidium. These data are interpreted as reflecting two conformational isomers of the IVS that differ in their reactivity and in their sensitivity to dye binding.

INTRODUCTION

The ribosomal RNA (rRNA) in *Tetrahymena thermophila* is transcribed as a 35S precursor RNA, which is processed to form the mature 17S, 5.8S and 26S rRNAs. The earliest identified step in this processing is the removal of a 413 nucleotide intervening sequence (IVS) from the 26S rRNA sequence by RNA splicing (1-4). Splicing occurs by a series of transesterification reactions involving the addition of a nonencoded guanosine to the 5' end of the IVS, release of the IVS, and ligation of the exons (5). These reactions can occur *in vitro* in the absence of any enzymes or other proteins (6).

The excised IVS is capable of further reactions. The 3' hydroxyl of the IVS RNA can make an intramolecular nucleophilic attack at the phosphate between nucleotides 15 and 16, releasing a 15 nucleotide fragment containing the 5' end of the molecule and forming a circle (7). A minor product is formed by nucleophilic attack at the phosphate between nucleotides 19 and 20, releasing a 19 nucleotide fragment (8,9).

The circular form of the IVS RNA retains catalytic activity. It undergoes hydrolysis at a specific bond to form a linear IVS 15 nucleotides shorter than the original excised IVS (8). The cyclization reaction can also be reversed by adding an excess of the 15 nucleotide fragment released during cyclization (Sullivan, Walstrum, Uhlenbeck and Cech, unpublished results). These reactions indicate that the cleavage-ligation activity is intrinsic to the IVS RNA and that the conformation of the IVS RNA is important in mediating these reactions.

The secondary structure of the IVS RNA has been extensively probed by enzymatic digestion and chemical modification under nondenaturing conditions, and the results have been interpreted with the aid of computer modeling (10, 11). The structure is remarkably similar to that obtained by others for *Tetrahymena* and *Physarum* nuclear rRNA introns and for various fungal mitochondrial mRNA and rRNA introns, based on consensus sequences found within these RNAs and on computer modeling (12-15). The structural similarities among these group I introns suggest that they may have a common origin and may undergo similar processing reactions. It is now known for several of these other systems that a non-encoded guanosine is added to the 5' end of the IVS (16,17) and that the RNA is self-splicing *in vitro* (18,19).

One strategy for studying the relationship between the structure and the function of the *Tetrahymena* IVS RNA involves the use of intercalating dyes, which are known to alter RNA secondary structures (20,21). Since the IVS RNA has a readily measurable activity, we can look selectively at that subset of dye binding that inhibits reactivity. We find that IVS activity and reaction specificity are remarkably resistant towards intercalating dyes, but that reactivity does sharply decrease at high dye concentrations. This work provides the necessary framework for investigating the sites of dye binding, described in the accompanying paper.

MATERIALS & METHODS

Materials

Ethidium bromide was purchased from Sigma, proflavine (3,6-diamino-acridine-HCl) from Aldrich, and acridine orange-HCl from Eastman. They were used without further purification. One ethidium preparation showed significant ribonuclease activity and was replaced with a different lot. Dyes were dissolved in sterile doubly distilled water and stored at -20°C. No difference was found between freshly prepared ethidium and ethidium stored at -20°C for several months. Dye concentrations were determined on a Cary 219 spectrophotometer using the extinction coefficients determined by Hudson and Jacob (22)

and by Weill and Calvin (23). Phenylalanyl tRNA and H-EPPS (N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid) were purchased from Sigma.

Ethidium is known to bind to eppendorf tubes, which can significantly alter the amount of free ethidium in solution at the lower concentrations (24; J. Nelson, personal communication). No difference was found between using ethidium-washed tubes and tubes which were siliconized with a solution of 4 ml dichlorodimethylsilane and 76 ml CCl_4. However, unsiliconized and unwashed eppendorf tubes did show variability; the extent of cyclization was sometimes less than that obtained using siliconized tubes. Small amounts of added ethidium (1 - 2 μM) often caused an increase in reactivity in these cases.

Preparation of end-labeled IVS

Earlier precursor RNA preparations were transcribed *in vitro* using pIVS11 plasmid DNA and *E. coli* RNA polymerase as described previously (6,10). Later preparations were transcribed *in vitro* using pJK43-SP6 plasmid DNA (cut with Eco RI) and SP6 polymerase (25,26). Transcription was usually carried out in 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 4 mM spermidine, 10 mM DTT, 1 mM NTPs and 100 - 200 μCi [^{3}H]-UTP (ICN) at 30°C for 2 - 4 hours. Little splicing was found to occur under these conditions. Unincorporated nucleotides were removed by chromatography on a Sephadex G50-150 column.

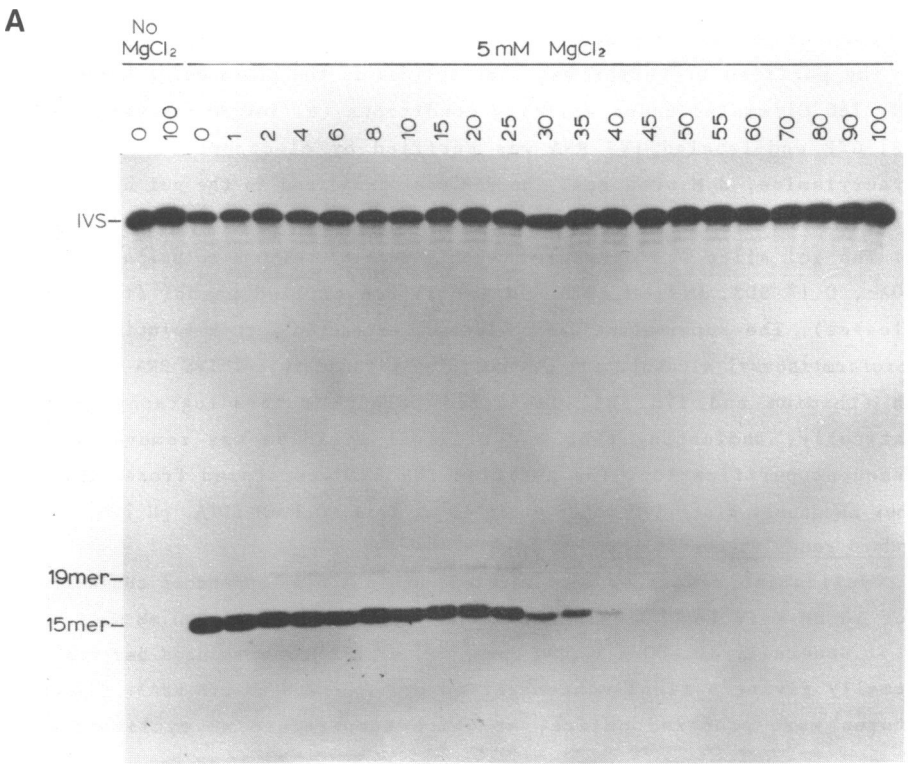
The purified precursor was then spliced in the presence of [α-^{32}P]-GTP (NEN, 760 Ci/mmole) under splicing conditions (6) for 30 minutes at 30°C. [^{32}P]-GTP end-labeled IVS RNA was purified by electrophoresis in a 4% polyacrylamide, 8 M urea gel. The RNA was visualized in the gel by ethidium staining or autoradiography, and the band corresponding to the IVS was cut out. The gel slice was extracted overnight at 4°C with a solution of 500 mM NH_4OAc, 0.1% SDS, 0.1 mM EDTA and 1 - 2% redistilled phenol (to inhibit nucleases). The supernatant was collected, extracted with a solution of 24:1 chloroform:isoamyl alcohol, and ethanol precipitated twice. IVS RNA visualized with ethidium and IVS RNA visualized through autoradiography behaved identically, indicating that the original ethidium was removed during subsequent purification. The purified IVS RNA was stored frozen at -20°C either in doubly distilled water or in 10 mM Tris, 0.1 mM EDTA, pH 7.5.

Standard reaction conditions

Cyclization reactions were carried out in 1.5 ml eppendorf tubes in 10 μl of 10 mM Tris (or 10 mM H-EPPS), pH 7.5, 0.1 mM EDTA, 200 mM NaCl, 5 mM $MgCl_2$. Generally 10,000 - 20,000 cpm [^{32}P] of IVS RNA were used per reaction (normally giving a final concentration of 0.5 - 2.0 nM IVS RNA). Reactions mixtures were prepared on ice, at which temperature no cyclization is

observed. Reactions were carried out at 42°C for 20 minutes. They were then quenched on ice, and 10 µl of a solution of 10 M urea, 20% sucrose, 0.02% xylene cyanol, 0.02% bromophenol blue, 0.5% SDS, 10 mM Tris base, 8.3 mM boric acid, 0.1 mM EDTA was added. Samples were heated to 65°C for several minutes and then loaded directly on a 20% polyacrylamide, 7 M urea sequencing gel (0.5 mm thick and 20 cm long). The running buffer was 100 mM Tris base, 83 mM boric acid, 1 mM EDTA.

Electrophoresis was stopped when the bromophenol blue was at the bottom of the gel. The gel was exposed to Kodak XAR 5 film (X-Omat AR) at -70°C with a DuPont Cronex intensifying screen, generally for 4 and 12 hours. Using the autoradiogram as a guide, the bands corresponding to the IVS, the 15mer, the 19mer and occasionally the 26mer were cut out and extracted with 400 µl NEN Protosol overnight at 37°C. The samples were neutralized with 50 µl glacial acetic acid, and 4 ml of fluor containing 2 parts 16.0 g 2,5-diphenyloxazole, 0.30 g p-bis(o-methylstyryl)benzene (Eastman) in 4 liter Scintanalyzed toluene with 1 part RPI Triton X-100 was added. Samples were counted in a Beckman



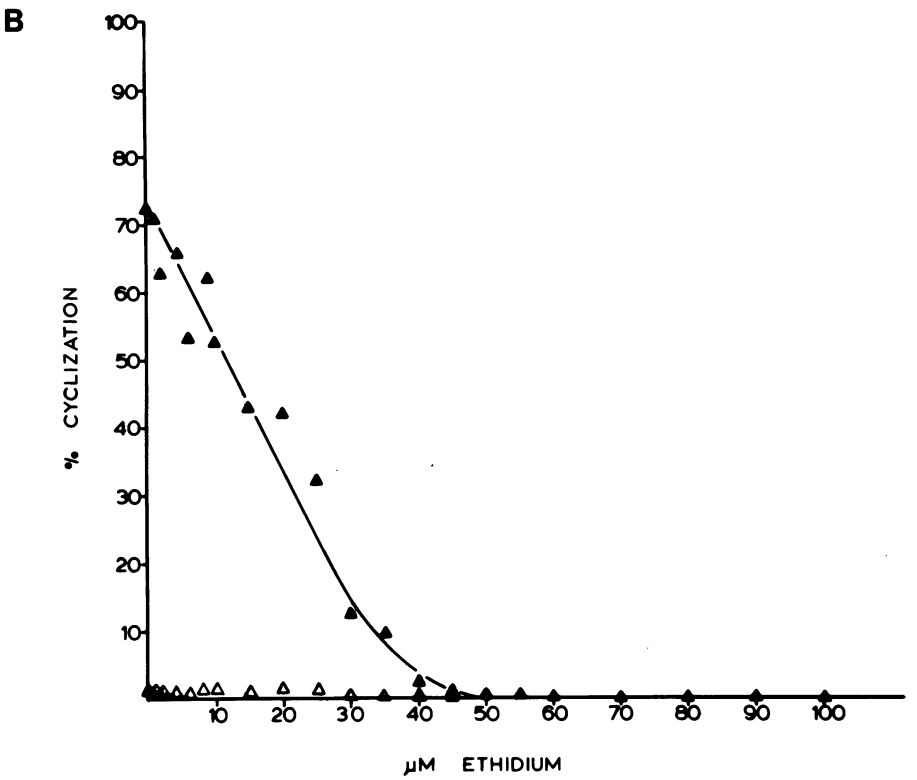


Figure 1. Ethidium bromide inhibits the cyclization reaction. (A) 5' end-labeled IVS RNA was incubated under normal cyclization conditions in the presence of 0 - 100 μM ethidium (lanes 3 - 23). The intensity of the bands corresponding to the 15mer and 19mer progressively decreases with increasing ethidium. In the absence of $MgCl_2$ no products are seen (lane 1) and no ethidium-specific degradation occurs (lane 2). (B) The percentages of 15mer (▲) and 19mer (Δ) produced during cyclization were determined as described in Materials & Methods and plotted against ethidium concentration.

LS7000 scintillation counter.

Little degradation was found for the different dye concentrations as indicated by the uniformity of the counts recovered from each gel lane. Normally 60% of the original counts were recovered. Background counts were subtracted from each band and the counts in the band were expressed as a percentage of the total counts isolated from that lane. The degree of cyclization after 20 minutes in the absence of dye under these conditions was normally about 83% (81 ± 6% 15mer; 2 ± 1% 19mer).

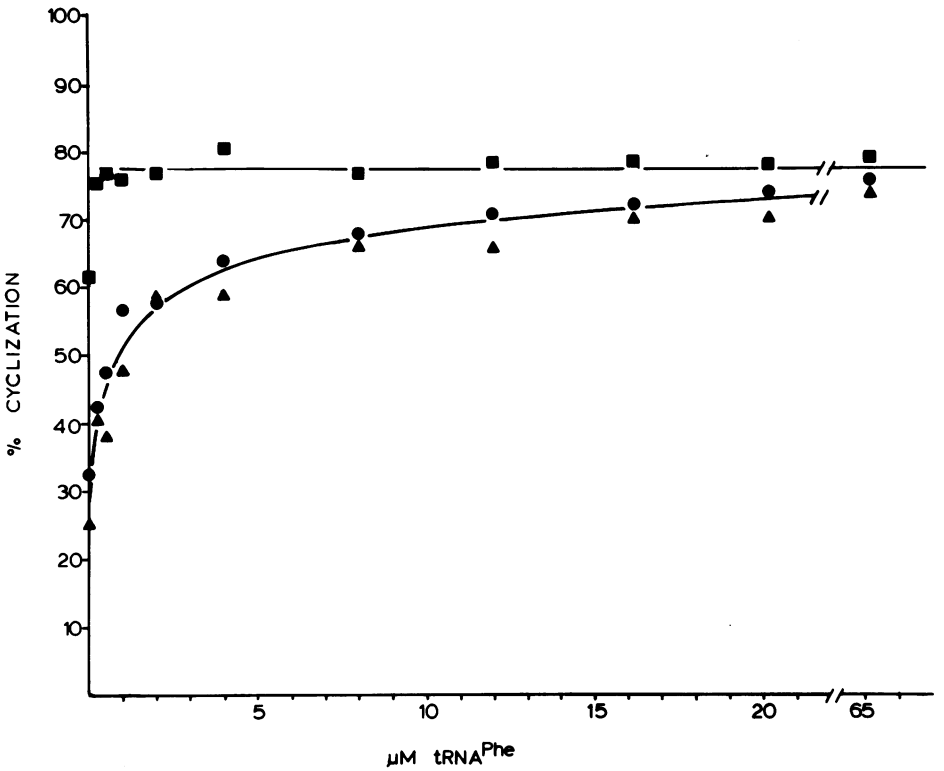


Figure 2. Ethidium inhibition is reversible. Increasing concentrations of unlabeled $tRNA^{Phe}$ progressively reduce the level of inhibition of cyclization by 20 μM ethidium. The IVS RNA was pre-equilibrated with the ethidium prior to adding the carrier (▲) or the carrier was pre-equilibrated with the ethidium prior to adding the IVS RNA (●). $tRNA^{Phe}$ by itself (■) had no affect on cyclization. Only the percentage of 15mer produced is shown, but the 19mer showed the same trend.

RESULTS

Ethidium inhibits the cyclization reaction

During the cyclization reaction, a 15 nucleotide or 19 nucleotide fragment is released from the 5' end of the IVS (7-9). If the IVS RNA is 5' end-labeled, these fragments retain the label and are a direct measure of cyclization. This is seen in lane 3 of Figure 1A, where the linear IVS, the 15mer and the 19mer are apparent on the autoradiogram, whereas the unlabeled circle is not.

Lanes 4 - 24 of Figure 1A show that increasing concentrations of ethidium progressively inhibit the cyclization reaction. The results are quantitat-

ed in Figure 1B. From the average of thirteen such experiments, one half inhibition was found to occur at 22 ± 14 μM ethidium. Complete inhibition (greater than 99%) occurred by 53 ± 16 μM. Inhibition was independent of the IVS RNA concentration (from 0.5 nM to 76 nM) and independent of the ethidium preparation (data not shown). The variability in the level of ethidium inhibition seems to reflect differences between IVS RNA preparations, since within any particular experiment the results were generally quite consistent. This is supported by the observation that the degree of cyclization in the absence of inhibitors was also variable between preparations.

Generally there was an increase in the percentage of 19mer observed at intermediate ethidium concentrations. Normally the increase was less than 30% over the amount of 19mer produced in the absence of dye, but sometimes the increase was as much as 100 - 150%. This is a further indication of the variability between IVS RNA preparations, and it suggests that there are multiple IVS RNA conformations that vary in their reaction specificity and activity.

Ethidium inhibition is reversible

To determine if ethidium inhibition is due to dye binding and not due to some dye-specific IVS RNA modification, we tested the reversiblity of inhibition. Excess unlabeled carrier RNA or DNA was added to compete out ethidium molecules bound to the radioactively labeled IVS RNA. Figure 2 shows that increasing amounts of $tRNA^{Phe}$ progressively reduced the extent of inhibition by 20 μM ethidium. The order in which the reagents were added was found to be unimportant, indicating that the ethidium was in rapid equilibrium with the RNAs. In the absence of ethidium, $tRNA^{Phe}$ had no effect on the cyclization reaction.

For this experiment, one half reversal of ethidium inhibition occurred at about 1 μM tRNA. Virtually complete recovery of cyclization activity was obtained in the presence of 65 μM tRNA. The $tRNA^{Phe}$ was effective at lower concentrations than would be anticipated if each tRNA molecule was binding only 3 - 4 ethidium molecules with an affinity of $1 \times 10^5\ M^{-1}$. However, the data were consistent with the tRNA simply reducing the concentration of ethidium free in solution. The amount of carrier RNA required for reversal is, of course, dependent on the extent of the original inhibition. Hind III digested phage λ DNA also reduced ethidium inhibition (data not shown).

Ethidium is a kinetic inhibitor

The IVS RNA was incubated with increasing concentrations of ethidium for three different times: 20 minutes (standard reaction time), 60 minutes and 180

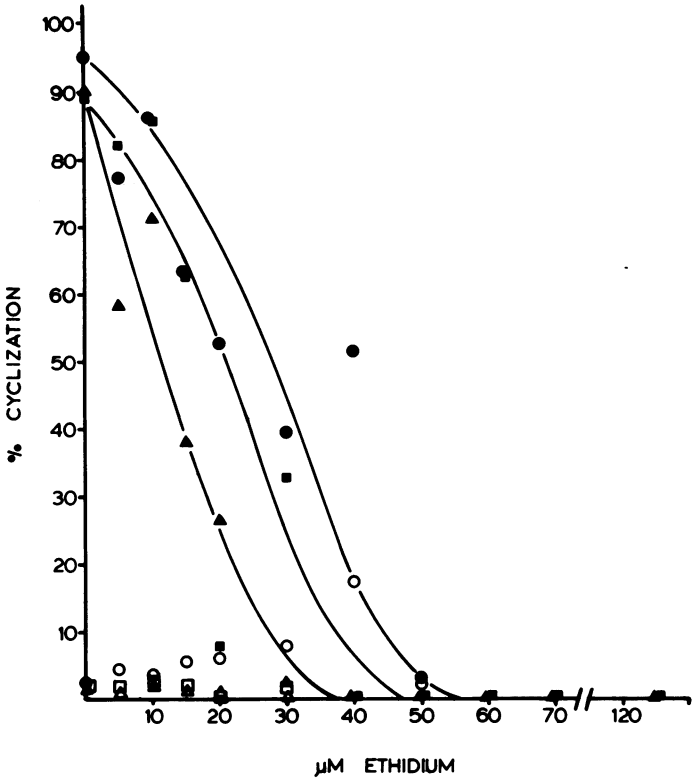
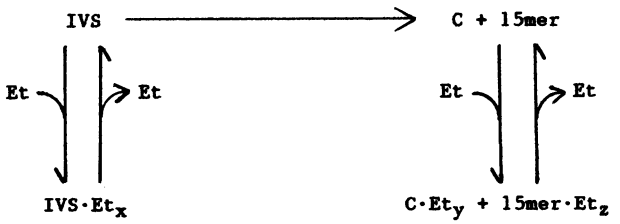


Figure 3. Ethidium is a kinetic inhibitor of cyclization. The extent of cyclization is shown as a function of increasing ethidium concentration. The IVS RNA was incubated under cyclization conditions for times of 20 minutes (▲), 60 minutes (■), and 180 minutes (●). Solid symbols, 15mer; open symbols 19mer.

minutes. Although IVS RNA degradation increased with the longer incubation times, only ~7% fewer counts were recovered at 180 minutes than at 20 minutes. As shown in Figure 3, one half inhibition increased from ~13 μM for a 20 minute incubation to ~27 μM for 180 minutes. This experiment was repeated with the same results (data not shown). These data indicate that ethidium is kinetically inhibiting the cyclization reaction as shown below:

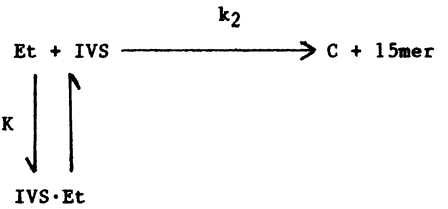


where IVS is the linear IVS RNA, C is the circular IVS RNA, and Et is ethidium bromide. This diagram shows that the ethidium is in equilibrium with the RNA reactants and products, but that the cyclization reaction proceeds in only one direction at these RNA concentrations. Thus, at subsaturating ethidium concentrations we would expect all the IVS RNA to cyclize if given sufficient time.

The relative amount of 19mer formed increased with the longer incubation times, suggesting that those molecules inhibited from cyclizing at the major site could still cyclize at the minor site. Alternatively, there could be a mixed population of IVS RNA conformations that differ in their reactivity and in their ethidium sensitivity.

The IVS RNA is remarkably resistant to inhibition

Because of the lack of equilibrium conditions between the RNA reactant and products, we could not use standard thermodynamic formulations to calculate the ethidium binding constant to the IVS RNA, or to estimate the number of ethidium binding sites involved in the inhibition. However, we were able to make an estimate based on kinetic calculations. This was done by first assuming a single binding site which, when occupied by ethidium, totally inhibits cyclization:



where K is the association constant for binding of ethidium to the IVS RNA, and k_2 is the rate of the cyclization reaction. If ethidium is assumed to be in great excess, then the association constant can be expressed as:

$$K = \frac{[IVS \cdot Et]}{[Et]o[IVS]} = \frac{[IVS]o - [IVS]}{[Et]o[IVS]}$$

where [IVS]o and [Et]o are the initial linear IVS RNA and ethidium concentrations, respectively. This equation can then be rearranged and expressed as a rate of product formation:

$$\frac{dC}{dt} = \frac{k_2([IVS]o - [C])}{K[Et]o + 1}$$

If this equation is integrated from 0 to C, product, and 0 to t, time, then:

$$\left[\ln \frac{[IVS]o}{[IVS]o - [C]}\right]^{-1} = \frac{K[Et]o}{k_2 t} + \frac{1}{k_2 t}$$

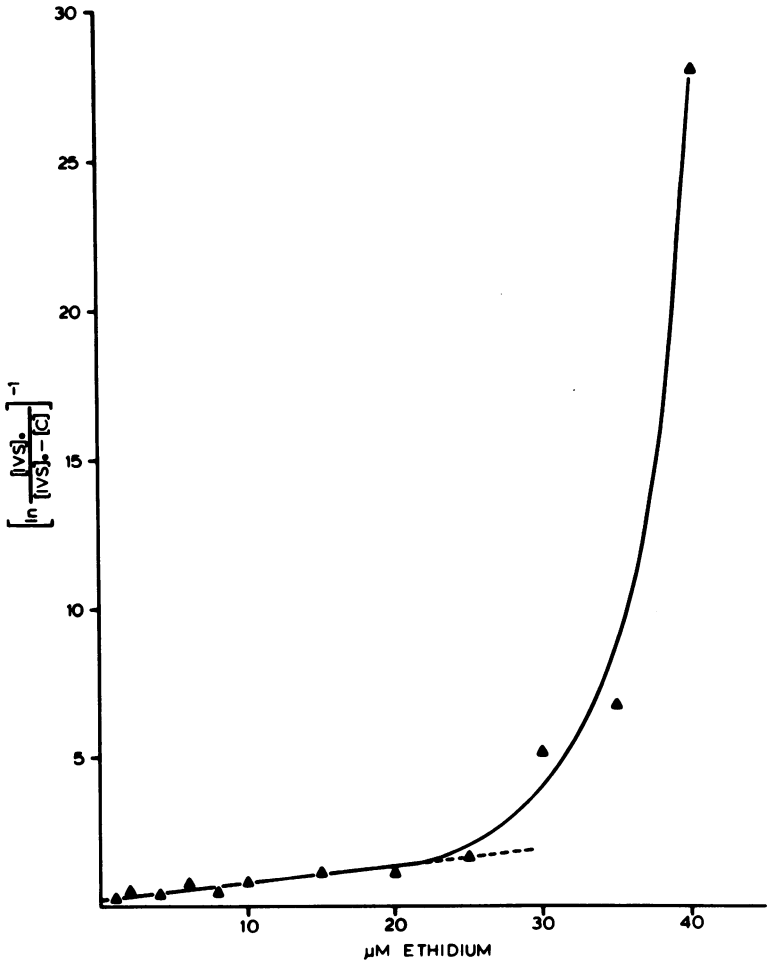


Figure 4. Kinetic analysis of the ethidium inhibition curve in Fig. 1B. The data were analyzed by the equation derived in the text. For this calculation, the percentage of 15mer and 19mer formed were added for each ethidium concentraton and then normalized to give 100% cyclization at 0 μM ethidium. Thus, only the reactive IVS RNA molecules were included in the calculation. Not all the data points from Fig. 1B are shown.

Thus, a plot of $[\ln([IVS]o/([IVS]o - [C]))]^{-1}$ versus [Et]o should give a straight line with a slope of K/k_2t and a y-intercept of $1/k_2t$ for a single site inhibitor. The data of Figure 1B is plotted in this manner in Figure 4. A line can be drawn through the initial data points; the results give an association constant $K = 2.0 \times 10^5\ M^{-1}$. For comparison, the measured binding constants for ethidium bound to tRNA are $5 \times 10^5\ M^{-1}$ to $1 \times 10^6\ M^{-1}$ for the

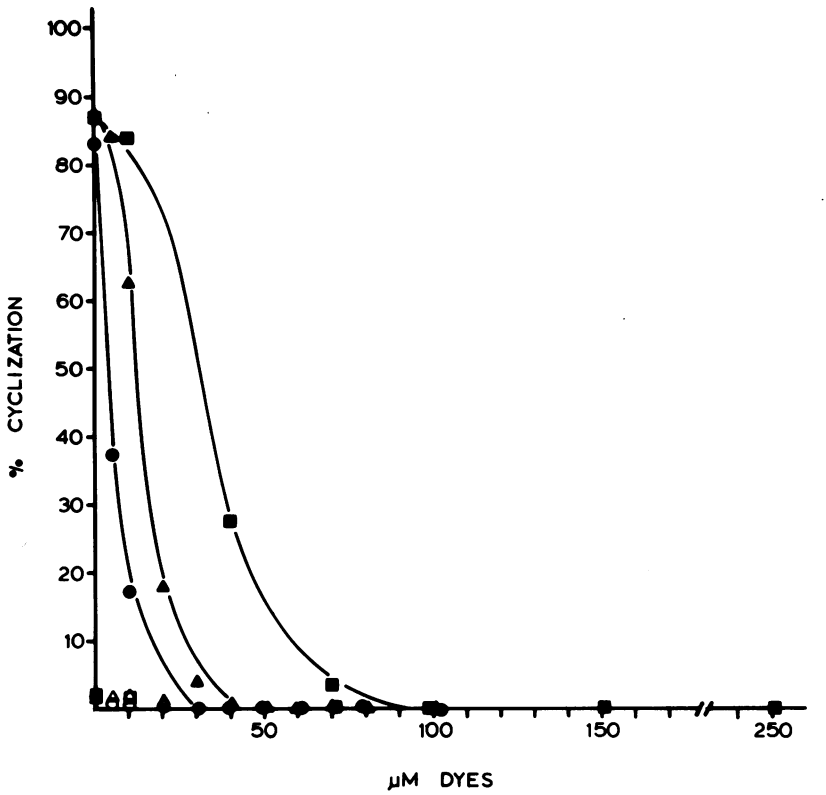


Figure 5. Other intercalating dyes inhibit cyclization. The effect of acridine orange (●) and proflavine (■) on the cyclization reaction are compared with that of ethidium (▲). Solid symbols, 15mer; open symbols, 19mer.

strong binding sites (27-29) and 5 X 10^4 M^{-1} for the weaker associations (27) at ionic strengths similar to those used in these experiments. The value of k_2 determined from the plot (0.2 min^{-1}) is also in the range expected for cyclization. Thus, we can at least approximate the inhibition curve at low ethidium concentrations by a single-site inhibitor. However, the full range of data cannot be fit with a single ethidium binding site. Therefore, it seems likely that inhibition is due to the additive or cooperative effects of more than one IVS RNA bound ethidium molecule or due to a mixed population of IVS RNA conformations with different sensitivities to ethidium.

These calculations only address the ethidium molecules that inhibit cyclization. We have no measure of the molecules bound to the IVS RNA that do not affect cyclization. However, the total number of bound molecules can be rough-

ly calculated by assuming an association constant of 2 X 10^5 M^{-1} and 100 independent intercalative binding sites, which is the maximum number of sites available if the IVS RNA is 100% base-paired and half of these sites are available to bind an ethidium (neighbor exclusion principle). We estimate that over 80% of the potential binding sites are occupied at ethidium concentrations required for one half inhibition. Over 50% of the potential sites are occupied if an association constant of 5 X 10^4 M^{-1} is assumed. So, while the experimental evidence suggests that inhibition is due to the additive or cooperative effect of more than one IVS-bound ethidium molecule, it seems likely that ethidium bound to most of the sites has little or no effect on the reaction. This would explain why methidiumpropyl-EDTA·Fe(II), an analog of ethidium that is capable of cleaving nucleic acid chains at its site of binding, cuts weakly at every residue of the IVS RNA (see accompanying paper).

Other intercalating dyes are inhibitory

Inhibition by acridine orange and proflavine are compared to inhibition by ethidium in Figure 5. The results show that acridine orange and ethidium are more effective inhibitors than proflavine. Both acridine orange (30) and ethidium (31) are known to have stronger binding constants to DNA than proflavine, under ionic strengths similar to those used in these experiments. This further suggests that inhibition of the cyclization reaction is simply a result of the dyes binding, probably intercalatively, to the IVS RNA. However, it is also known that these dyes display variations in their binding properties (20,21), so other factors may account for some of the differences in effectiveness observed between dyes. It is unlikely that these three dyes share any common contaminants, so inhibition seems to be intrinsic to the intercalating dyes.

Cation concentration affects ethidium inhibition

Because both the IVS RNA structure and its reactivity are dependent on the concentration of Mg(II) and monovalent cations, the salt dependence of ethidium inhibition was examined. In general, higher $MgCl_2$ concentrations shifted the inhibition to higher ethidium concentrations (Fig. 6). Increasing the $MgCl_2$ concentration from 5 mM to 10 mM was found, in this and other experiments (not shown), to increase the concentration of ethidium required for one half inhibition by 2 - 3 fold. With 20 mM $MgCl_2$, in the presence or absence of NaCl, there was an initial strong level of inhibition which then leveled off to a final extent of inhibition smaller than observed at lower $MgCl_2$ concentrations. In the absence of added NaCl, 5 mM and 10 mM $MgCl_2$ gave similar inhibition curves (1/2 inhibition at 12 μM ethidium; data not shown).

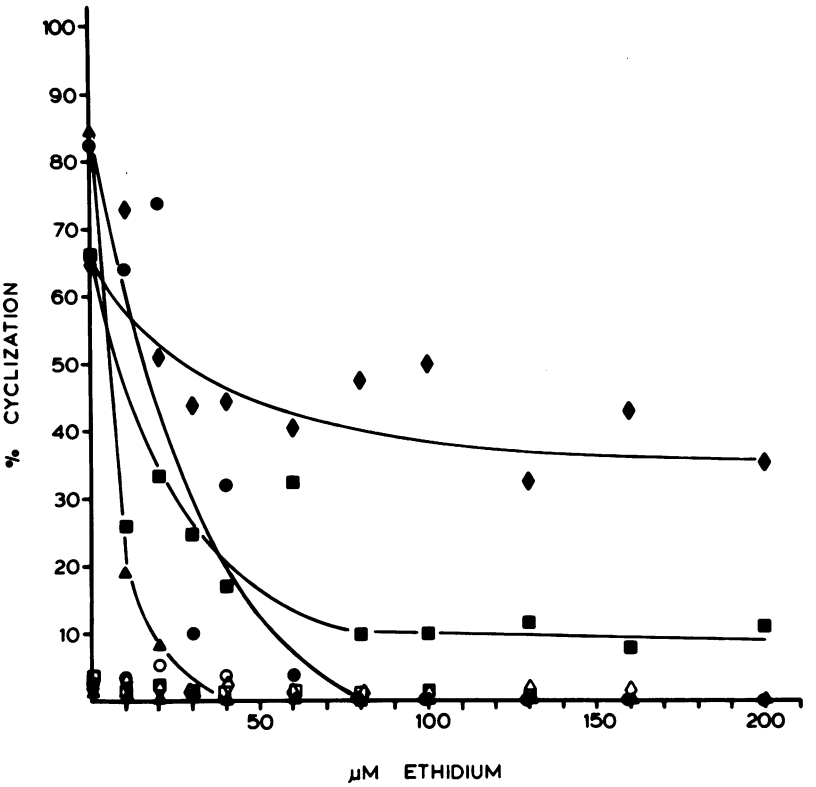


Figure 6. Ethidium inhibition is sensitive to the ionic conditions in the reaction buffer. The percentage of 15mer (solid symbols) and 19mer (open symbols) are shown as a function of ethidium concentration with reaction buffers containing: 5 mM $MgCl_2$, 200 mM NaCl (normal reaction buffer) (▲); 10 mM $MgCl_2$, 200 mM NaCl (●); 20 mM $MgCl_2$, 200 mM NaCl (■); and 20 mM $MgCl_2$ (♦). This series should result in increasing stabilization of the IVS RNA, as reflected by an increased melting temperature (44,45).

The percentage of 19mer also increased with increasing $MgCl_2$ concentrations, and a new oligonucleotide product appeared. It corresponded in size to the 26mer described previously (9) and amounted to as much as 2% of the total counts at 10 μM ethidium, 20 mM $MgCl_2$ and 200 mM NaCl.

$MgCl_2$ is also a kinetic inhibitor

Figure 6 shows that there is ~25% less cyclization at 20 mM $MgCl_2$ in the absence of ethidium, than at 5 mM $MgCl_2$. Longer incubation times at high $MgCl_2$ concentrations do not show this reduction in cyclization (data not shown). Thus it seemed likely that Mg(II) was acting as a kinetic inhibitor of

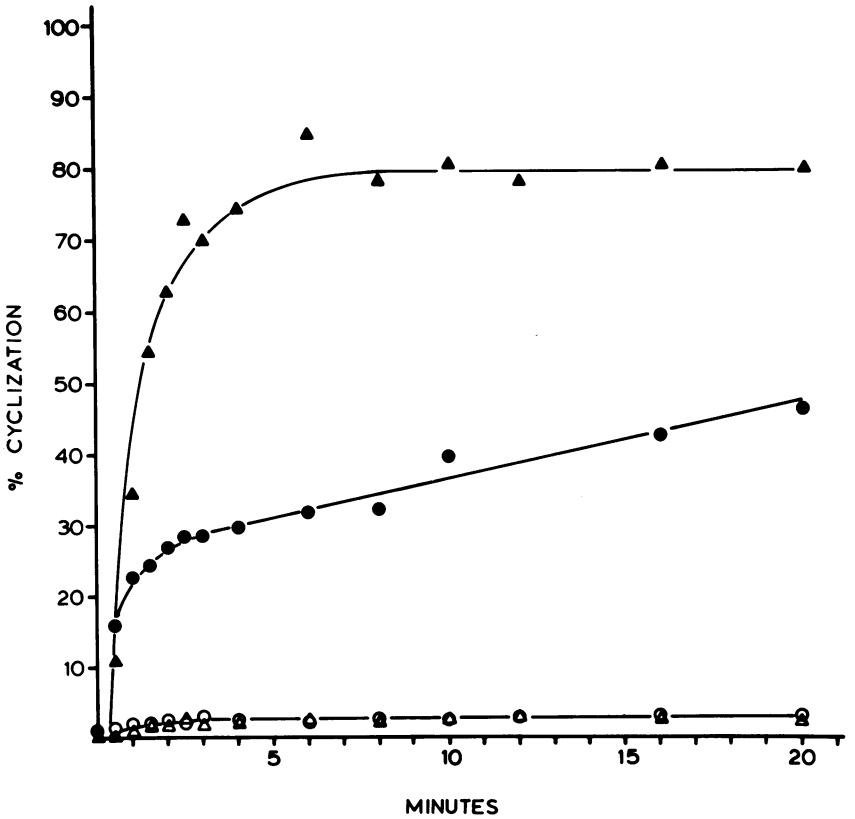


Figure 7. High concentrations of Mg(II) ions kinetically inhibit cyclization. The percentage of 15mer (closed symbols) and 19mer (open symbols) were measured as a function of time under normal cyclization conditions, but with 10 mM $MgCl_2$ (▲) or 50 mM $MgCl_2$ (●) in the reaction buffers.

the reaction. We tested this by analyzing the cyclization rate in the presence of 200 mM NaCl and either 10 mM or 50 mM $MgCl_2$. As shown in Figure 7, the cyclization reaction occurs very rapidly (1st order kinetics) in the presence of 10 mM $MgCl_2$, with one half reaction occurring by approximately 1 minute. The initial lag in the reaction probably represents the time required for the samples to heat to 42°C after being placed in the water bath. A 0.5 - 1 minute half-time is consistent with that determined previously (9). With 50 mM $MgCl_2$, the kinetics show a biphasic curve with an initial rate similar to that obtained for 10 mM $MgCl_2$, and a second, slower reaction rate. For the experiment shown in Figure 7, the fast kinetic component included about 25% of the

potentially reactive molecules. This proportion seems to vary significantly between IVS RNA preparations.

DISCUSSION

Dye binding affects reactivity, not fidelity

Binding of ethidium bromide, acridine orange or proflavine to the IVS RNA affects reactivity at the normal cyclization sites (positions 15 and 19). No new reaction sites are created. There is an increase in reaction at position 19 relative to position 15 at intermediate dye concentrations, but this is a relatively minor increase in relation to the total products. Similarly, another type of structural perturbation -- deletion of a portion of the IVS -- results in a reduction or elimination of activity, rather than alteration in the reactive sites (25). Only when the major cyclization site is mutated have new sites been found (Been and Cech, manuscript submitted).

Mechanism of dye inhibition

The fact that inhibition is reversible and is not intrinsic to a single dye clearly indicates that noncovalent dye binding to the IVS RNA is the source of the inhibition. Although the data do not reveal the actual mechanics of the dye inhibition, it seems likely that inhibition is due mostly to intercalative dye binding, rather than to weaker coulombic binding, which is less preferred at the high salt concentrations used in these experiments (32). The inhibitory strength of the three dyes seems to correspond to their strong intercalative binding constants. This, of course, does not preclude the possibility of the dyes binding strongly to regions of the IVS RNA in a non-intercalative fashion as has been observed for ethidium binding to tRNA (33), or to differences due to the binding properties of the individual dyes.

The narrow range of dye concentrations required for inhibition indicates that multiple binding sites are involved, with binding constants in the range expected for dyes bound to tRNA ($\sim 1 \times 10^5\ M^{-1}$), and that binding to these sites has an additive or cooperative effect on the inhibition. It is unlikely that inhibition could be due entirely to dye binding to the hypothesized active site, where the 3' terminal G and the three pyrimidines preceeding the cyclization site are bound, since such binding would be expected to give a single-site inhibition curve.

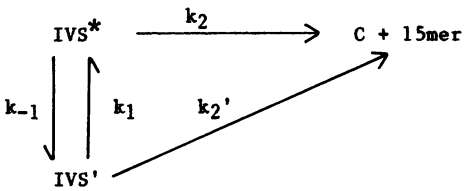
Intercalating dyes are known to alter the structure of nucleic acids by elongating, stiffening and unwinding helical regions (20,21). They are also known to cause helical transformations; ethidium induces conversion of three-stranded helices formed from poly(rA) and poly(rU) to two-stranded helices

(34) and also induces helical changes that bind ethidium more efficiently (34-37). Dyes are thought to kink or bend helical regions at their site of intercalation (20,38), but are also known to stabilize helical structures as reflected by an increased melting temperature (20,21). Mismatches and bulged out regions in nucleic acid helices are also stabilized by dyes (39,40).

Thus dyes may act to inhibit cyclization by disrupting secondary or tertiary interactions necessary for reactivity. Alternatively, dyes may stabilize structural features of the IVS RNA, thus slowing down or preventing conformational changes necessary for the reaction. This latter model may also explain the inhibitory effect of high levels of $MgCl_2$, although the actual details of the inhibition are undoubtedly different.

Conformational dynamics

The data on the kinetic inhibition of cyclization at high $MgCl_2$ concentrations are difficult to explain without invoking two conformational isomers of IVS RNA that have different reactivity. One possible explanation is that there are two or more IVS RNA conformations, only one of which is reactive. With low $MgCl_2$, the various conformations are in rapid equilibrium. With high $MgCl_2$, the various forms are stabilized and the rate of interconversion is reduced; thus the first part of the plot in Figure 7 represents the initial reactive molecules, while the second part is indicative of the rate of change from the inactive to the active form:



where $k_2' = 0$, IVS' is the inactive linear IVS RNA form(s) and IVS* is the active form. In this model, k_2 is the rate limiting step at low $MgCl_2$, but k_1 becomes the rate limiting step at high $MgCl_2$ concentrations. Another explanation is that these different conformations display different reaction rates. Thus under low salt conditions, the interconversion between conformations is rapid and k_2 is again the rate limiting step. The slow reaction, k_2', is not seen. At high $MgCl_2$ concentrations, isomerization between these conformations (k_1, k_{-1}) may be blocked or greatly reduced, so only the slow reaction rate, k_2', is seen at the longer incubation times.

Interestingly, the formation of 19mer under high $MgCl_2$ conditions does not parallel the results obtained for the 15mer (Fig. 7). Instead it yields the same pattern as obtained with low $MgCl_2$; that is, there is a fast initial

reaction that levels off to a constant value early in the time course. This suggests that the production of 19mer is dependent on a IVS RNA species that is distinct from that which forms the 15mer, and that these forms may not readily interconvert.

Ethidium inhibition was strongly dependent on the $MgCl_2$ concentration (Fig. 6). Mg(II) ions are known to strongly reduce ethidium binding to DNA and RNA (41). This reduction of the binding affinity, at least for the primary sites, is caused by a decrease in the strength of the interactions rather than a change in the number of sites available (42). This decrease in binding affinity readily explains the shift to higher ethidium concentrations necessary for one half inhibition in going from 5 mM $MgCl_2$ to 10 mM $MgCl_2$ in Figure 6. The curves obtained with 20 mM $MgCl_2$ in the presence or absence of 200 mM NaCl are more complex. These curves also seem to consist of two IVS RNA classes, but with different ethidium sensitivities. Since the concentration of the ethidium-resistant species is dependent on the absolute $MgCl_2$ concentration, we must also propose that high Mg(II) concentrations favor ethidium-resistant conformation(s).

Comparison with previous studies

Nielsen *et al.* (43) attributed proflavine inhibition of Tetrahymena rRNA splicing *in vivo* to dye-specific disruption of transcriptional termination. The aberrantly long transcripts were thought to be incapable of efficient splicing. While we do not directly address the *in vivo* situation, accurate termination has not been found to be critical for correct splicing *in vitro* (6). It seems likely from our studies that inhibition of splicing and of termination could be independent effects of the dyes.

ACKNOWLEDGEMENTS

We are grateful to David Draper for help with the kinetic calculations, to Carol Cech, Cammy DeLuca-Flaherty and Dennis Prosen for providing *E. coli* RNA polymerase, to Arthur Zaug for providing SP6 polymerase, to Jeff Nelson for helpful information, and to Stan Gill and Doug Turner for many useful discussions. This work was supported by American Cancer Society grant NP-374 and by the Biomedical Research Support Grant Program, Division of Research Resources, NIH. T.R.C. was supported by a Research Career Development award from the National Cancer Institute, Department of Health and Human Services.

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