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Studies on transcription termination and splicing of the rRNA precursor *in vivo* in the presence of proflavine

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ABSTRACT

In isolated nucleoli from Tetrahymena thermophila, low concentrations of the intercalating agent proflavine inhibit both transcription termination and splicing of the rRNA precursor. Proflavine also exerts an in vivo effect on the process of transcription termination under conditions, where the growth rate is only slightly reduced. Thus, approximately 40% of the rRNA precursor molecules, accumulated in nucleoli during 60 min of treatment with the drug, are longer than the normal 35S rRNA precursor. R-Loop mapping of these longer precursor molecules isolated after 30 and 60 min of incubation demonstrates that the RNA polymerases have a 50 fold lower elongation rate in the spacer region than in the coding region. Proflavine in the given concentration is found to have no significant effect on the splicing of properly terminated precursor molecules. In contrast, none of the longer non-terminated molecules are found to be spliced. These results indicate that proflavine primarily affects the process of transcription termination and that the splicing event is inhibited due to the improper termination of the precursor molecule.

INTRODUCTION

Base-paired secondary structures are found in heterogenous nuclear RNA of various types of cells [1-5]. As double-strand specific RNases are found to be involved in site specific cleavage of RNA, it has been suggested that double-strand regions might be of importance for the processing of RNA precursor molecules[6]. Also the observation that various intercalating agents increase the metabolic stability of nuclear RNA precursors implicates the involvement of such base-paired structures in the regulation of cleavages of the precursor RNA molecule [7-10].

We have previously demonstrated that low concentration of intercalating agents inhibit both RNA splicing[11] and transcription termination [12,13] in isolated nucleoli, indicating that

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double-stranded RNA structures are also involved in the regulation of these processes. In the present series of experiments we have by RNA-DNA hybridization and R-loop mapping studied the *in vivo*-effect of proflavine. The results show a direct effect on the termination process, while the influence on the splicing process seems to be a consequence of lack of correct terminations.

Based on these results we discuss data by others on the effect of proflavine and imply that the increased metabolic stability of the RNA precursor molecules also in these cases primarily might have been caused by the effect of the drug on the termination process.

METHODS

Culture conditions. *Tetrahymena thermophila* (strain B 1868-7) was grown in a complex medium to a density of 60,000-80,000 cells per ml as previously described[13]. Cultures were treated with proflavine at concentrations ranging from 10-50 µg per ml of culture for 0, 30, and 60 min, respectively. Cells were harvested and nucleolar chromatin isolated as nucleoli as described before[14].

RNA size studies were done on 1.8% agarose gels containing 5 M urea by the method of Dudov *et al.*[15] in the presence of marker RNAs.

Southern hybridization. rDNA was digested with Hind III restriction endonuclease and electroforesed on 2% agarose gels. The DNA was blotted onto nitrocellulose filters as described by Southern[16], and hybridized to ^{32}P-labelled rRNA synthesized in isolated nucleoli in the absence and presence of 30 µg proflavine per ml [11].

R-Loop formation. Nucleic acids from isolated nucleoli were extracted with phenol-chloroform-isoamylalcohol and precipitated with ethanol. The pellet containing rDNA and rRNA was dissolved in 20 mM Tris-HCl pH 7.4, made to 70% formamide, 100 mM NaCl, 10 mM EDTA, and 100 mM HEPES pH 7.8, and incubated at 70°C for 15 min in order to denature the rDNA. Hybridization was performed at 41°C for 16-18 h[11].

The R-loops were stabilized by fixation with glyoxal for 2

h at 13°C according to the method af Kabach et al.[17]. The hybridization mixture was diluted into 50% formamide, 100 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA and spread in the presence of cytochrome c (0.1 mg/ml) on a hypophase of 20% formamide, 10 mM Tris-HCl pH 7.4. The protein films were picked up on parlodion coated grids, stained with uranyl acetate[18] and shadowed with platinum-palladium. Electron micrographs were taken with a Siemens Elmiskop 101 at 60 KV. The length of the molecules was measured with a Hewlett Packard 9864B digitizer connected with a 9810 calculator. All lengths were normalized to an average size of the snap-back molecule of 10,000 bp[11].

Dot-blot analysis of rRNA. Nucleic acids were isolated by homogenization of nucleoli in guadinium buffer (4 M guanidinium isothiocyanate, 40 mM Tris-HCl pH 7.5, 8 mM EDTA, 80 mM 2-mercaptoethanol, 4% sarkosyl). The homogenate was made to 0.9 M in CsCl and layered on a 1 ml cushion of 5.7 M CsCl, 100 mM EDTA in a Beckman SW60 polyallomer tube. Pelleting was performed by centrifugation for 17 h at 37,000 rpm at room temperature. The RNA samples were denatured at 65°C, spotted on nitrocellulose filter, previously wetted with water and soaked from 30 min in 20XSSC, dried under a heat lamp and baked at 80°C for 6 h. The RNA containing filters were prehybridized and then hybridized to ^{32}P-labelled cloned rDNA probes as described in ref. 19. The hybridization probes, plasmid pGY19 (named pI, cf. Figure 1), pRP4 (pII) and pRP7 (pIII) all originate from Dr. Ron Pearlman.

RESULTS

In Tetrahymena the rRNA genes are located on multiple extra-chromosomal, palindromic rDNA molecules[20] (Figure 1). The genes can be isolated with the associated chromosomal proteins as transcriptionally active nucleoli[14]. Transcription of such nucleoli leads to the synthesis of a transcript with the size of 6.2 kb (Figure 2A, lane 1). The presence of proflavine during the transcription on the isolated chromatin cause an increase in the size of transcript to 8.2 kb (Figure 2A, lanes 2-5). This indicates a deletion of the transcription termination signal whereby the endogenous RNA polymerases read into the distal spacer region (cf. Figure 1 and ref.21). Hybridization of rRNA

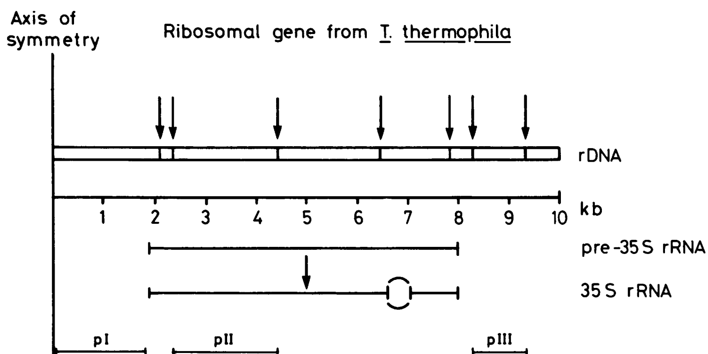

Figure 1. Map of the rRNA gene from Tetrahymena thermophila based on Engberg et al.[20], Çarin et al.[11] and Engberg, personal communication. The cleavage sites for the restriction endonuclease Hind III are indicated by arrows. pI, pII and pIII are the rDNA probes used for the dot-blot analysis of rRNA (cf. Figure 3).

synthesized in the absence and presence of proflavine to Hind III-restriction fragments of rDNA verify this observation. Thus, only rRNA synthesized in the presence of proflavine hybridizes to the 1.05 and 0.65 kb fragments originating from the distal spacer region (Figure 2B). Proflavine in the same concentrations also inhibits splicing of the primary transcript in the isolated nucleoli as previously described[11].

In order to investigate the in vivo-effect of proflavine on both the process of transcription termination and splicing, RNA-DNA hybridization and R-loop studies were performed on total rRNA of nucleoli isolated from proflavine-treated cells. For that purpose exponentially growing cultures were treated with proflavine at concentrations of 10-50 µg/ml. This range of concentrations of proflavine was chosen as it has only a slight effect on the general cellular processes measured over a period of one generation. Thus, the growth rate and the RNA synthesis were reduced by less than 20%. Dot-blot analyses of isolated total nucleolar rRNA from proflavine treated and untreated cells demonstrate the existence of a class of non-terminated rRNA molecules in proflavine treated cells. Figure 3 shows the results of such an experiment. rRNA was spotted in four different amounts (0.3, 1.0, 3 and 9 µg, respectively) on nitrocellulose filters and hybridized to ^{32}P-labelled rDNA representing: (A)

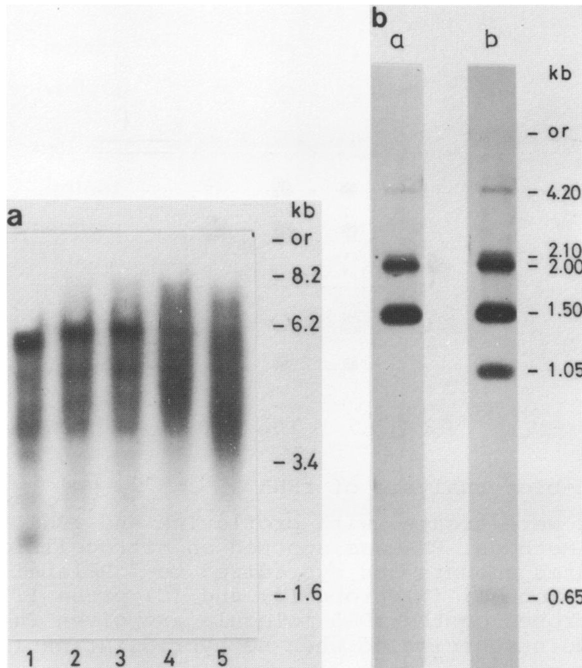

Figure 2. The effect of proflavine on the size of the transcript synthesized on isolated nucleolar chromatin

A. Samples of nucleoli were incubated in a ^{32}P-labelled assay mixture for 30 min at 25°C in the presence of proflavine: (1) 0 μg/ml; (2) 10 μg/ml; (3) 20 μg/ml; (4) 30 μg/ml; (5) 40 μg/ml.

B. Autoradiogram of Hind III restriction fragments of rDNA after hybridization to ^{32}P-labelled rRNA synthesized in isolated nucleoli (a) in the absence, (b) in the presence of 30 μg/ml of proflavine.

the non-transcribed central spacer (pI, cf. Figure 1), (B) the coding region (pII) and (C) the non-transcribed distal spacer (pIII). rRNA from control cells does hybridize only to the probe from the coding region (Figure 3B), while rRNA from proflavine-treated cells also hybridizes to the probe representing the distal spacer (Figure 3C). This demonstrates that proflavine causes the RNA-polymerase molecules to read through the normal termination signal into the distal spacer. In contrast, proflavine is found to have no effect on the initiation properties, as rRNA from proflavine-treated cells does not hybridize to the probe representing the central spacer (Figure 3A).

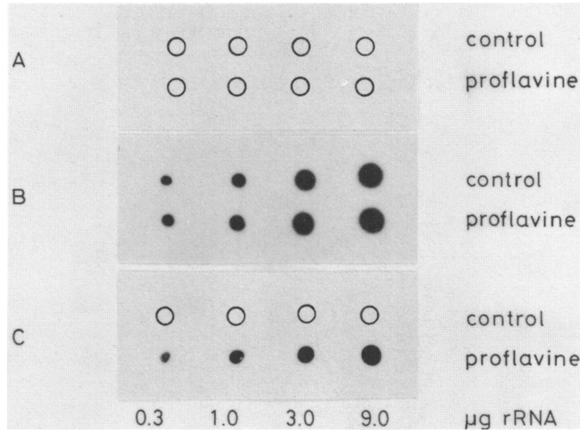

Figure 3. Dot-blot analysis of rRNA

Cell cultures were treated with proflavine and rRNA isolated as described in Methods. RNA was spotted on nitrocellulose filters in the indicated amounts and hybridized to ^{32}P-labelled probes of rDNA; (A) probe I, (B) probe II, and (C) probe III. The position of the probes on the rDNA molecule are given in Figure 1. Application points encircled when no hybridization is observed.

The rRNA was characterized by electron microscopic analysis of R-loop molecules formed by hybridization of total nucleolar rRNA to snap back molecules of rDNA. Two distinct classes of rRNA molecules were observed in nucleoli from untreated cells: (1) non-spliced pre-35S rRNA (Figures 4A), and (2) spliced 35S rRNA (Figure 4B). The same two types of rRNA molecules are found by analysis of total nucleolar nucleolar rRNA from proflavine treated cells. However, in addition a new class of non-spliced molecules composed of non-terminated rRNA molecules is observed (Figure 4C). These molecules are all longer than the normal precursor due to transcription of the distal spacer region. The quantitative results of the R-loop analysis of the total nucleolar rRNA content are given in Table 1. In untreated cells, 24% of the molecules are present as spliced rRNA precursor molecules, while the remaining 76% exist as non-spliced precursor molecules. In nucleoli of cells treated with proflavine for 30 or 60 min, spliced precursor molecules represent 32 and 31%, respectively, indicating that proflavine in the given concentration has no significant effect on the splicing process.

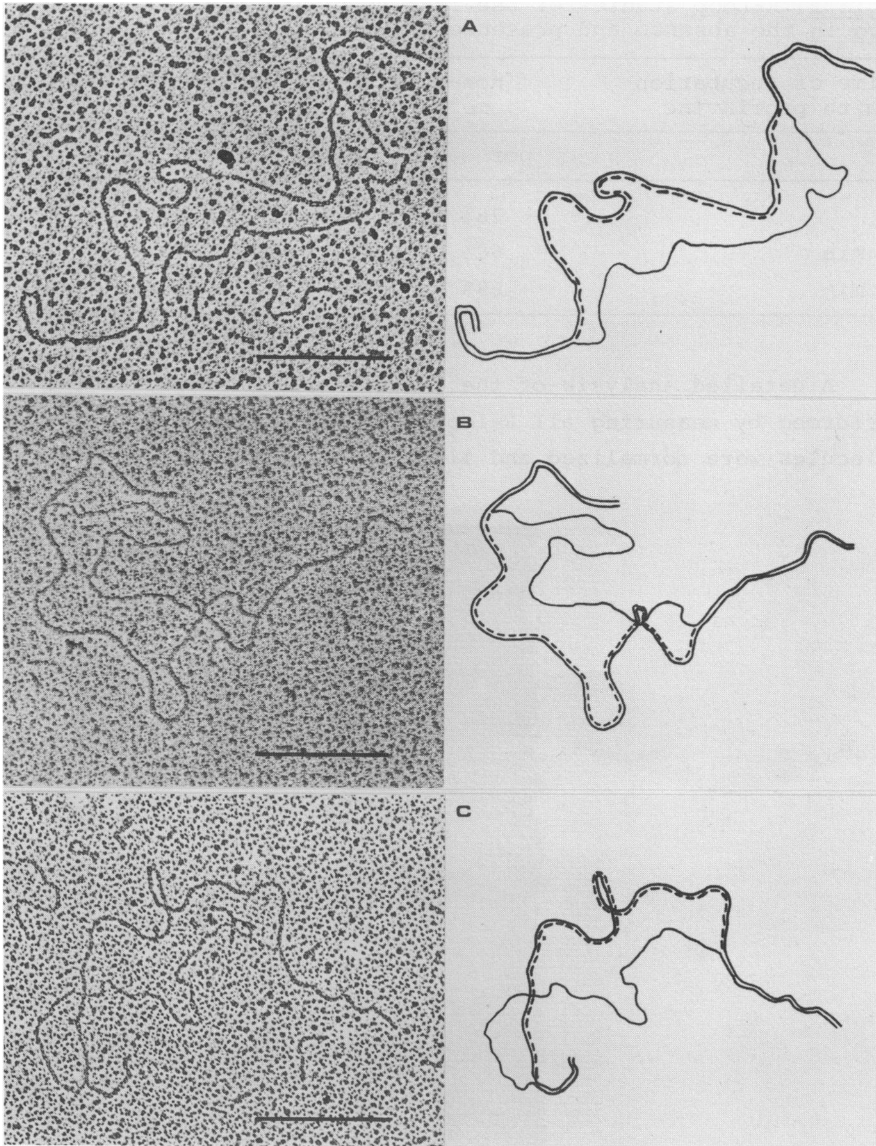

Figure 4. Electron micrographs of R-loop molecules

Nucleic acids from isolated nucleoli were isolated, hybridized under conditions where rDNA forms snap-back molecules and spread for electron microscopy. For details see Methods. The presented R-loop molecules are formed (A) with pre-35S rRNA, (B) with 35S rRNA, and (C) with non-terminated rRNA. In the schematic drawings, rDNA strands are symbolized by a solid line, rRNA by a dashed line. The bar represents 0.5 μ.

Table 1. R-Loop studies of the rRNA precursor synthesized *in vivo* in the absence and presence of proflavine (30 μg/ml).

Time of incubation with proflavine	non-spliced molecules		spliced molecules	
	per cent	(n)	per cent	(n)
0	76%	(62)	24%	(19)
30 min	68%	(74)	32%	(34)
60 min	69%	(107)	31%	(48)

A detailed analysis of the *in vivo*-effect of proflavine was performed by measuring all R-loop molecules. The registrated molecules were normalized and lined up by a computer programme

Figure 5. Computer outprint of R-loop molecules
The 107 listed molecules have arison from hybridization of rDNA with non-spliced rRNA precursor molecules from cells treated with proflavine for 60 min. The molecules have been normalized to a size of 10,000 bp and lined up in such a way as to give maximal similarity between the molecules in the series. DNA duplex stretches are designated by a thin line, R-loop stretches by a bar.

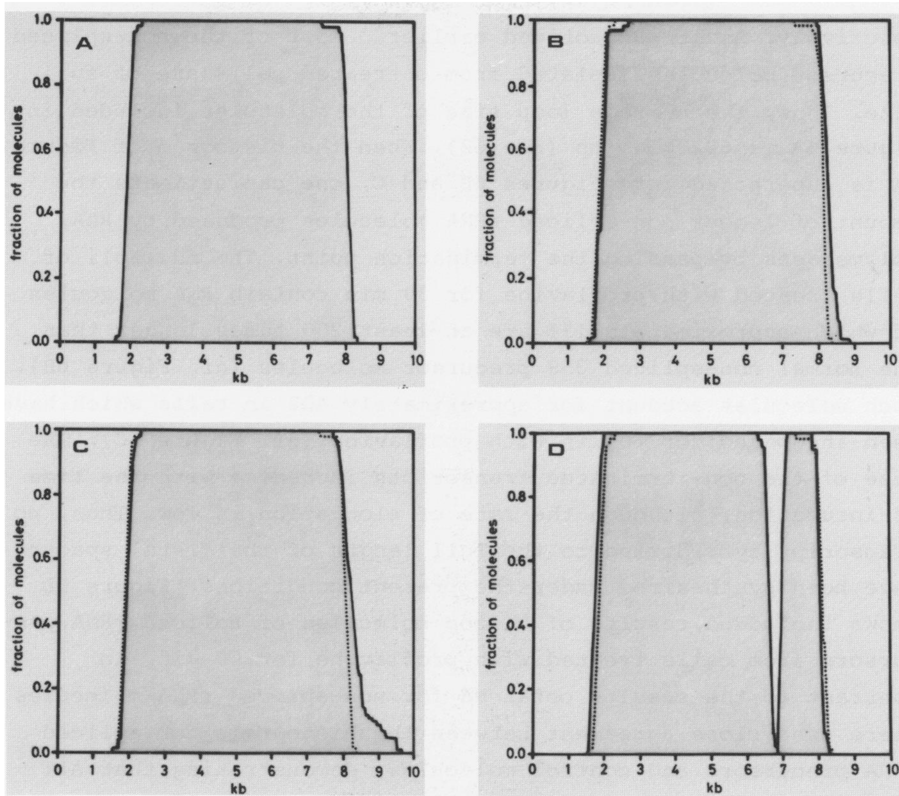

Figure 6. Added results of mapping of R-loop molecules

The histograms are based on the addition of R-loop molecules which have been listed as described under Figure 4. The solid profiles represent R-loop molecules including (A) non-spliced rRNA precursor molecules from control cells; (B) and (C) non-spliced rRNA precursor molecules from cells treated with proflavine for 30 and 60 min, respectively; (D) spliced rRNA precursor molecules from cells treated with proflavine for 60 min. R-Loop areas are shaded. The dotted line in B-D represents the superposion of histogram A.

in such a way as to give maximal similarity between the individual molecules in a series. As an example the computeroutprint of R-loop molecules representing non-spliced rRNA precursor molecules from cells treated with proflavine for 60 min is given in Figure 5. When the results from such outprints are added together a histogram is obtained. Figures 6A-C represent the added results for R-loop molecules of non-spliced rRNA precursors from cells treated with proflavine for 0, 30, and 60 min, re-

spectively. As it was noticed earlier[11] most of the non-spliced precursor molecules isolated from untreated cells are of full size. Thus, the average loop size of the molecules included in Figure 6A is 6220±240 bp (n = 62). When the histogram of Figure 6A is superposed into Figures 6B and C, one can estimate the amount of longer non-spliced rRNA molecules produced by RNA polymerases by-passing the termination point. The nucleoli of cells treated with proflavine for 30 min contain RNA molecules of which approximately 15% are at least 200 bases longer than the normal non-spliced 35S precursor molecules (cf. Figure 6B). Such molecules account for approximately 40% in cells which have been incubated for 60 min with proflavine (cf. Figure 6C). The size of the non-terminated transcripts increases with the time of incubation, although the rate of elongation is low. Thus, no transcript hybridizing to the full length of the distal spacer have been synthesized under the present conditions. Figure 6D shows the added results of R-loop molecules of spliced rRNA precursors from cells treated with proflavine for 60 min. In contrast to the results obtained for non-spliced rRNA molecules there is a close agreement between the histograms for spliced rRNA precursors and control molecules, demonstrating that all spliced molecules are correctly terminated.

DISCUSSION

Among several tested intercalating agents lucanthone and proflavine have been shown to exert a specific effect on the process of transcription termination in isolated nucleoli [12, 13]. In the presence of either of these drugs, the endogenous RNA polymerases on the isolated nucleolar chromatin read through the normal termination sequence and into the distal spacer, giving rise to a longer transcript than the normal 35S rRNA precursor. Proflavine also exerts an inhibitory effect on splicing of the *in vitro*-synthesized rRNA precursor[11], while lucanthone has only a slight effect on this process (unpublished results). The interaction of the drug with the two reactions indicates the involvement of double-stranded RNA structures in the processes, as previously suggested in a number of models for termination and splicing. The fact that the two drugs inhibit the reactions to

different extents, might suggest that different kinds of secondary RNA structures are involved in the reactions. A direct comparison of the sequences around the termination site and the splice junctions shows a dyad symmetry region at the termination site[22], while different secondary structures are formed at the splice junction and within the intron[23].

The present study shows that proflavine exerts a clear effect on the transcription termination process, when cultures of *Tetrahymena* are treated with low concentrations of the drug. Thus, treatment of cell cultures with 50 μg/ml of proflavine for 30 min results in accumulation of approximately 15% of non-terminated rRNA molecules. The fraction of non-terminated molecules increases to about 40%, when the cells are grown for 60 min in the presence of the drug. The prolonged incubation also gives a clear increase in the maximal size of the non-terminated transcript, although, in none of the endogenous RNA polymerases have read to the end of the distal spacer region.

From Figures 6B and C it can be estimated that the fastest RNA polymerase has transcribed 1000 bases of the distal spacer during the incubation period from 30 to 60 min. This is equivalent to a transcription rate of approximately 0.5 nucleotide per sec,which is extremely low compared to the estimated normal elongation rate of 25-40 nucleotides/second in the coding region (cf. ref.24). As proflavine in a concentration of 100 μg/ml has no effect on the elongation rate in the coding region *in vitro*[12], it seems plausible that the low *in vivo*-rate observed in the distal spacer region might be a consequence of the chromatin structure of this region. This is consistent with our earlier observation of a tight, well-defined nucleosomal-like structure of the distal spacer in contrast to the more open structure of the coding region[25]. It cannot be excluded based on the present data that random termination of the RNA polymerases in the distal spacer region does influence our estimation of the elongation rate. However, as both the size and number of non-terminated transcripts increase with the time of incubation, random termination will only have minor effect on this estimation.

Recently, experiments by Kruger *et al.*[26] have demonstrated

that *in vitro* splicing of transcripts which do not include the natural termination region of the pre-rRNA molecules can take place. The *in vivo* situation seems more complex[24], as S_1 studies by Engberg *et al.*[27] have shown that more than 20% of the nuclear pre-rRNA molecules of full length have not been spliced. The observation is in close agreement with our previous results[11] that more than 75% of the rRNA molecules associated with the nucleolar structure are full-length non-spliced molecules. Together these data indicate that the majority of the precursor molecules are terminated before splicing occurs. This is interesting since the splicing process has an estimated half lifetime of ~2 sec[24], while the RNA polymerases require 30-40 sec to transcribe the approximate 1100 nucleotides between the 3'end of the intron and the termination point. The present observation that none of the read-through transcripts get spliced under conditions where the normal percentage of correctly terminated molecules are spliced, indicate that proflavine has no direct effect on the splicing process *in vivo*. The effect is rather due to the presence of spacer sequences in the transcript alter the secondary structure of the splice site. Only a differential uptake of proflavine among the individual cells could explain the results if proflavine have a direct effect on the splicing process.

As previously mentioned, proflavine has in several cases been reported to increase the metabolic stability of various nuclear RNA precursors by inhibition of processing of these molecules [7-10]. Most of these reports are based on proflavine treatment *in vivo* followed by isolation and characterization of the RNA by methods which do not allow determination of the exact size or 3'end of the precursor molecules. Thus, it is possible that proflavine also in these cases primarily effects the termination event and thereby prevents processing of the transcript.

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