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# Syntheses of rRNA, 5.8S, 5S and tRNA are inhibited equally by 8-methoxypsoralen phototreatment of *Tetrahymena thermophila*

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## ABSTRACT

Treatment of the ciliated protozoan Tetrahymena thermophila with 8-methoxypsoralen combined with long wavelength ultraviolet irradiation (UVA, λ ~ 360 nm) resulted in a dose dependent equal inhibition of the synthesis of rRNA, 5.8S, 5S and tRNA. Similar results were obtained with 3-carbethoxy-8-methylpsoralen which predominantly forms DNA mono-adducts. In contrast the synthesis of tRNA in T. thermophila was much less sensitive than that of rRNA, 5.8S and 5S RNA to treatment with short wavelength ultraviolet irradiation (UVB, λ ~ 254 nm). These results are interpreted in favor of a mechanism by which psoralen-DNA adducts (crosslinks >> monoadducts) inhibit RNA transcription initiation (in contrast to UVB which causes premature chain termination). Furthermore it is argued that RNA synthesis is regulated in equally sized domains regardless of the gene-size.

## INTRODUCTION

Psoralens are naturally occurring phototoxic compounds that photoreact with the bases of DNA, especially thymine, forming cyclobutane adducts. Furthermore, some psoralens are capable of photoreacting with two adjacent thymines of opposite DNA strands thereby creating a DNA interstrand crosslink. It is generally accepted that the photobiological effects of the psoralens including toxicity, mutagenicity and carcinogenicity are primarily due to the formation of psoralen-DNA adducts (see 1-3 for recent reviews on psoralen photochemistry and photobiology). Accordingly, it has been shown that DNA synthesis and RNA synthesis is much more sensitive to psoralen-UVA treatment than protein synthesis (4-7).

The molecular basis for the impairment of DNA template activity by psoralen-adducts is not clear at present but studies on the photoeffect of the monofunctional isopsoralen, angelicin, on ribosomal RNA synthesis in monkey kidney cells (9) as well as in

vitro studies using psoralen adduct-containing DNA-templates as enzyme substrates (10-11) have suggested that chain termination at the adducts may be involved. If so the sensitivity to psoralen-UVA treatment of a certain gene should correlate with the size of its primary transcript. Supporting this hypothesis it has been found for mouse L-cells that the synthesis of 4S RNA is less sensitive to PUVA treatment than the synthesis of mRNA (12) and that the 28S/18S RNA ratio of newly synthesized RNA decreases after angelicin/UVA treatment of monkey kidney cells (9).

I now report that in the ciliated protozoan Tetrahymena thermophila, which has proven a very valuable model system for various processes in molecular biology (13), treatment with 8-methoxypsoralen-UVA results in parallel inhibition of 26S, 17S, 5.8S, 5S as well as tRNA.

## MATERIALS AND METHODS

8-Methoxypsoralen (puriss) was purchased from Fluka. 3-Carbethoxy-8-methylpsoralen was synthesized according to (14) and the identity and purity of the compound was ascertained by NMR spectroscopic as well as elemental analysis. Tetrahymena thermophila (strain B 1868-VII) was grown in complex proteose peptone medium and harvested in exponential growth phase ($10^5$ cells/ml) by centrifugation (500 g, 5 min). The cells were resuspended in 10 mM Tris-HCl pH 7.4 at $10^6$ cells/ml and 8-methoxypsoralen (or 3-carbethoxy-8-methylpsoralen) dissolved in 10 mM Tris-HCl pH 7.4 (diluted from a stock solution in dimethyl sulfoxide (10 mg/ml)) was added. Following an equilibration period in the dark (10 min) the cell suspension (2 ml) was irradiated from above through a 1 mm glass plate in a petri dish (1.8 $cm^2$) for 10 min (8 $kJ/m^2$) (Philips TL/09 fluoresecent light tube, 14 J x $m^{-2}$ x $s^{-1}$ at $\lambda \sim 365$ nm measured by ferrioxalate actinometry) and 10 vol of fresh medium was added. 1 $\mu$Ci of tritiated precursor ([$^3$H]-thymidine (50 Ci/mmol), [$^3$H]-uridine (30 Ci/mmol) or [$^3$H]-phenylalanine (46 Ci/mmol) was added to 1 ml aliquots for measurement of DNA, RNA or protein synthesis, respectively. The samples were incubated at 28$^{o}$C for two hours and the trichloroacetic acid precipitable radioactivity was measured. The synthesis of individual RNA species was measured by labeling of the psoralen-UVA treated cells ($10^6$ cells in 1 ml medium) with $^{32}$P inorganic phosphate (0.1 Ci/mg, 25 $\mu$Ci). RNA was

extracted from the pelleted cells with phenol either at 55°C (in 100 µl 50 mM sodium-acetate, 1 mM EDTA, pH 5, 0.5% SDS) or at 0°C (in 100 µl 1 mM $MgCl_2$, 3 mM $CaCl_2$, 70 mM NaCl, 25 mM Tris-HCl, pH 7.2, 1% SDS). The RNA was analyzed on 3% native polyacrylamide gels (0.16% bis-acrylamide, 70 mM Tris-phosphate, 2 mM EDTA, pH 7) or 10% denaturing polyacrylamide gels (0.33% bis-acrylamide, 7 M urea, 90 mM Tris-borae, pH 8.3, 1 mM EDTA). Labeled RNA was detected by autoradiography and quantified either by densitometric scanning of the autoradiograms or by cutting out the RNA bands (detected by ethidium bromide staining) from the gels followed by determination of the $^{32}P$-radioactivity.

Photographs of the ethidium bromide stained gels were scanned

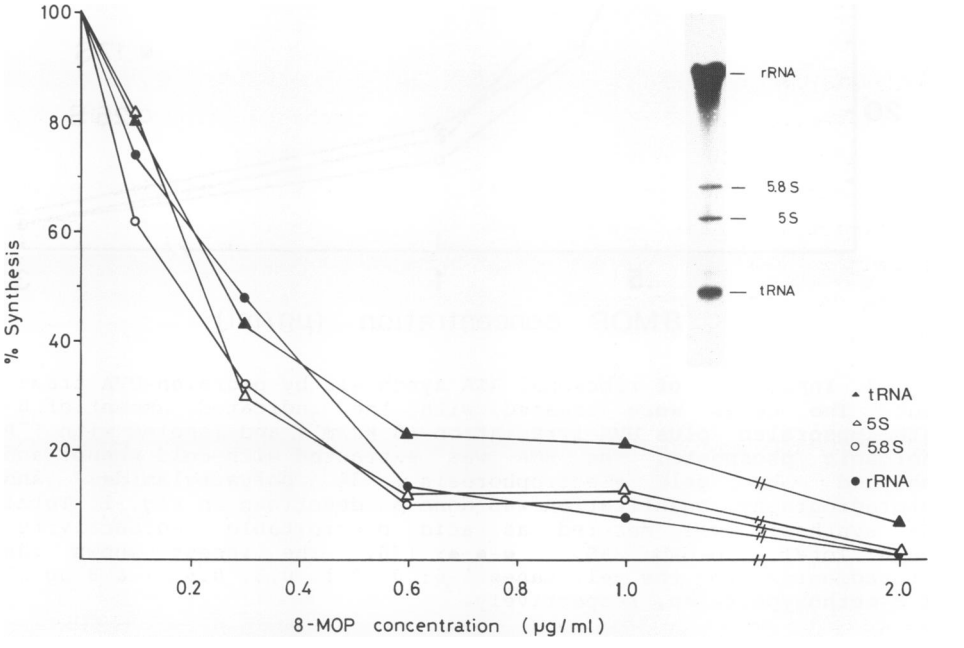



Fig. 1. Inhibition of individual RNA species by psoralen-UVA treatment. The RNA synthesis was measured by incorporation of $^{32}P$ inorganic phosphate. The RNA was extracted with hot phenol and analyzed by polyacrylamide gel electrophoresis (10%, denaturing) and autoradiography. (The insert shows the autoradiogram for controls without 8-methoxypsoralen). The quantitation of the individual RNAs was done either by densitometric scanning of the autoradiograms or by "Cerenkov-counting" of the excised RNA bands. The two methods gave indistinguisable results.●-●-●: rRNA, o-o-o: 5.8S, Δ-Δ-Δ: 5S, ▲-▲-▲: tRNA.

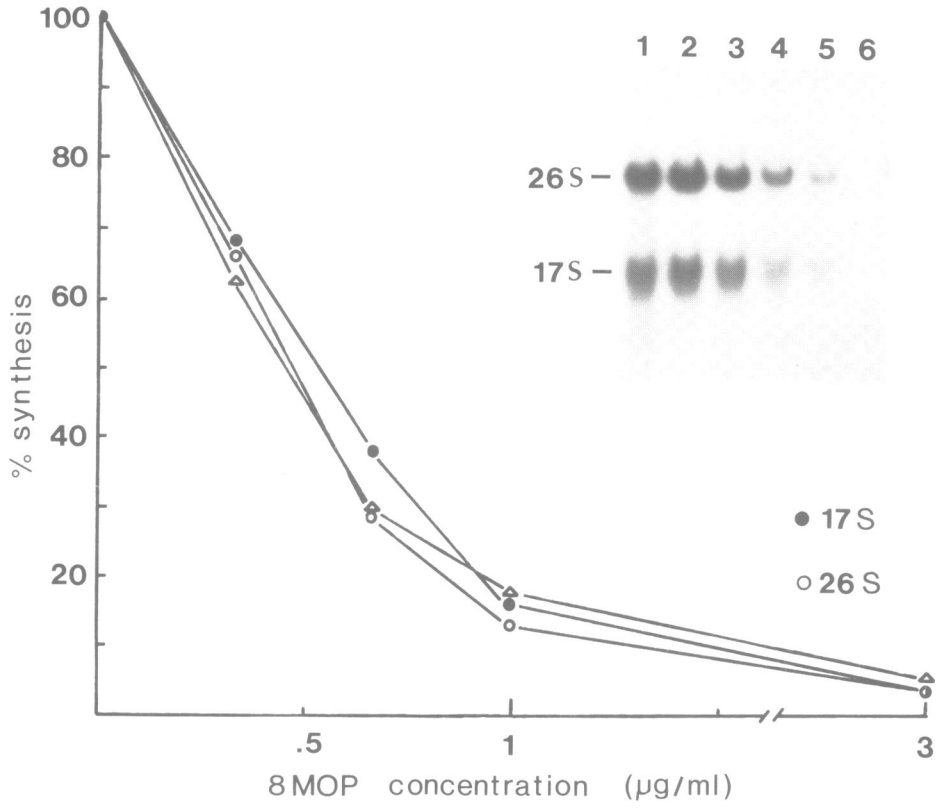



Fig. 2. Inhibition of ribosomal RNA synthesis by psoralen-UVA treatment. The cells were treated with the indicated amount of 8-methoxypsoralen plus UVA irradiation (8 KJ/$m^2$) and labeled with $^{32}P$ inorganic phosphate. The RNA was extracted with cold phenol and analyzed by gel electrophoresis (3% polyacrylamide) and autoradiography. Quantitation was done as described in Fig. 1. Total RNA synthesis was mesured as acid precipitable radioactivity. Δ-Δ-Δ: Total, o-o-o: 26S, ●-●-●: 17S. The insert shows the autoradiogram from the gel. Lanes 1-6: 0, 0.1, 0.3, 0.6, 1 & 3 µg/ml of 8-methoxypsoralen, respectively.

as well to ensure the reproducibility of the RNA extraction procedure and if required the results were corrected for uneven RNA amounts in the gels.

Irradiations with UVB light were done in 10 mM Tris-HCl pH 7.4 using two 15 W germicidal lamps at a distance of 30 cm (2 J · $m^{-2}$ · $s^{-1}$ measured by ferrioxalate actinometry). The cells (2 x $10^6$ in 2 ml) were kept a room temperature in a 1.8 $cm^2$ petri dish during the irradiation.

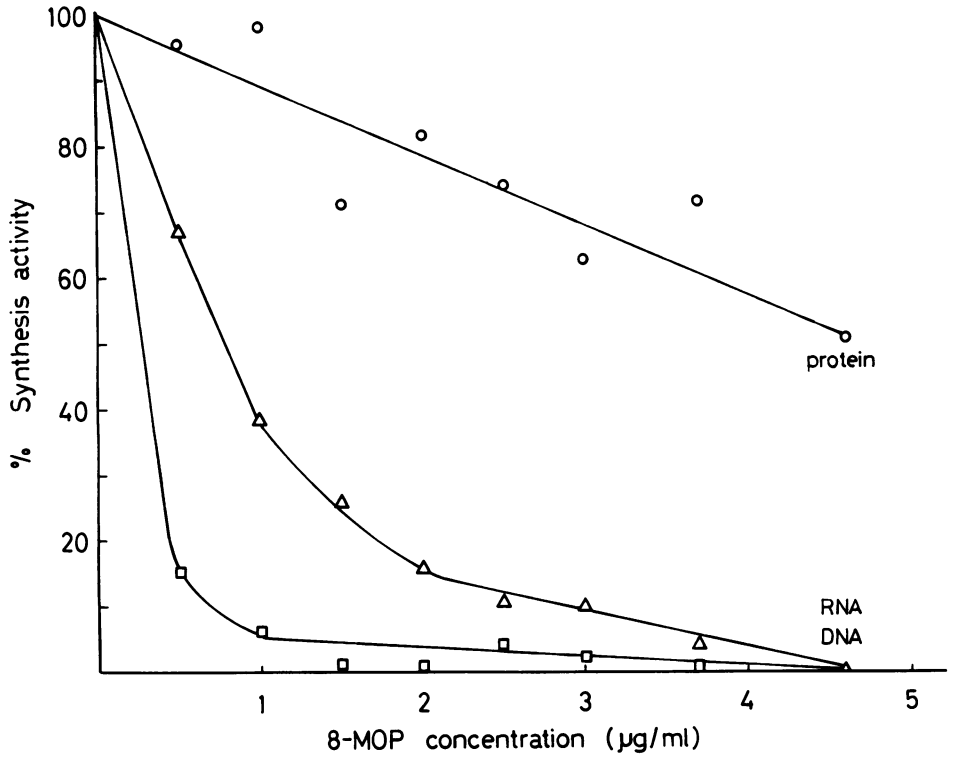



Fig. 3. Inhibition of DNA, RNA and protein synthesis following treatment of Tetrahymena thermophila with various concentrations of 8-methoxypsoralen plus long wavelength ultraviolet irradiation (8 KJ/m$^2$). o-o-o: protein, Δ-Δ-Δ: RNA, □-□-□: DNA.

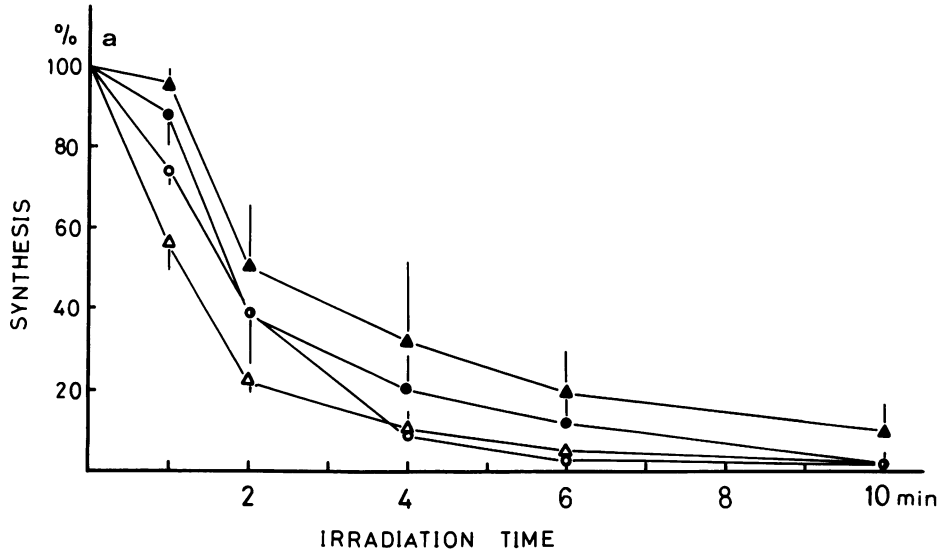
**RESULTS**

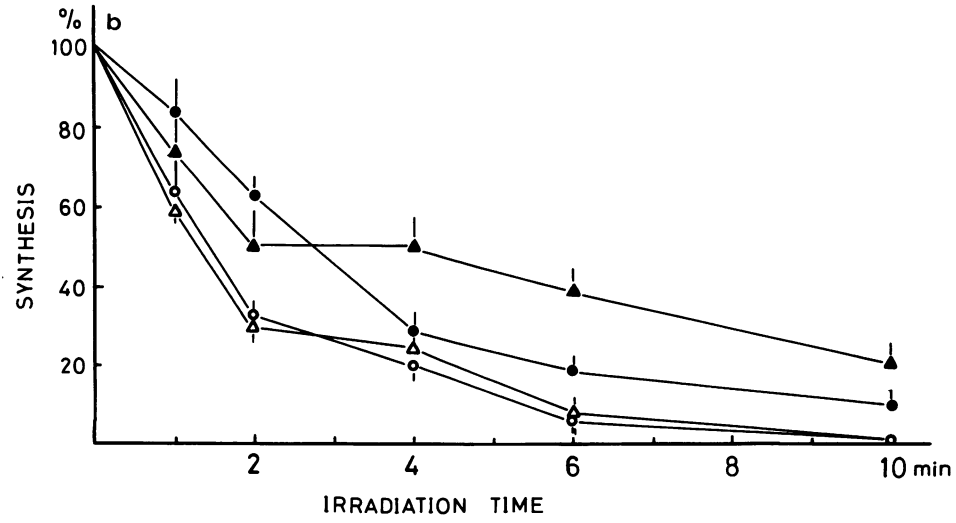
The incorporation of inorganic $^{32}P$ into ribosomal RNA, 5.8S, 5S and tRNA in Tetrahymena thermophila was measured after treatment with various concentrations of 8-methoxypsoralen using a fixed irradiation dose of 8 kJ/m$^2$. As shown in Figure 1 the synthesis of all RNA species are inhibited in parallel and thus are equally sensitive to the psoralen-UVA treatment. Any differential inhibition of the two ribosomal RNA species 17S and 26S RNA could not be detected either (Fig. 2). In order to ascertain that the observed effect was not due to a general toxicity to the cells whereby all cell functions were impaired, the synthesis of DNA, RNA and protein was measured in a separate experiment (Fig. 3). These results clearly show a preferential inhibition of DNA and RNA synthesis while

protein synthesis is much less affected, supporting previous findings that psoralen-UVA treatment causes specific impairment of DNA template functions (6-8).

Virtually equal inhibition of rRNA, 5.8S, 5S and tRNA synthesis was also observed when the irradiation dose was varied at constant 8-methoxypsoralen concentration (Fig. 4a).

The photoinhibitory effect on RNA synthesis of 3-carbethoxy-8-



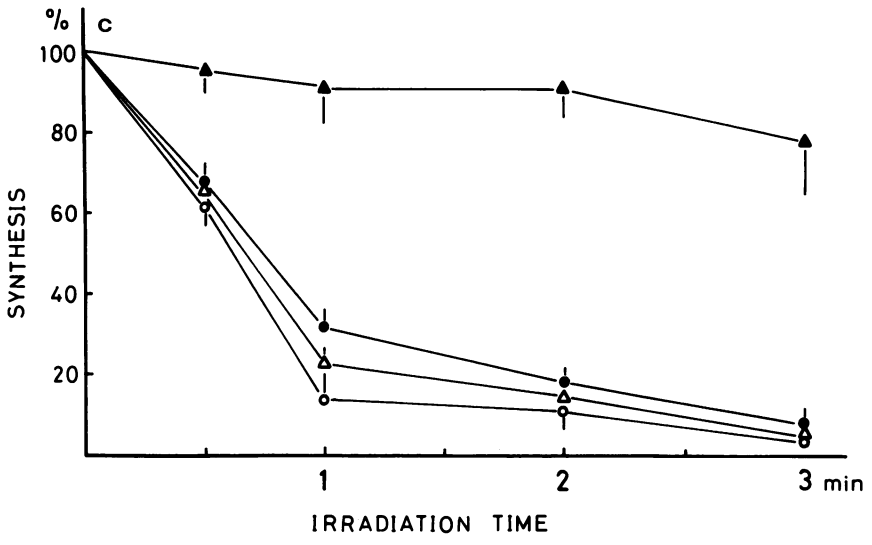



Fig. 4. Inhibition of the synthesis of rRNA (●), 5.8S (o), 5S (Δ) and tRNA (▲) in Tetrahymena thermophila treated with a) 8-methoxypsoralen (5 μg/ml), b) 3-carbethoxy-8-methylpsoralen (10 μg/ml) and long wavelength ultraviolet irradiation (UVA, λ ~ 365 nm) or c) short wavelength ultraviolet irradiation (UVB, λ ~ 254 nm). The cells ($10^6$/ml) were irradiated at room temperature in 10 mM Tris-HCl pH 7.4 for the times indicated and subsequently incubated ($10^5$/ml) at 28°C in the fresh medium containing $^{32}$P-phosphate (25 μCi/ml) for one hour. RNA was subsequently isolated and analyzed by polyacrylamide gel electrophoresis. Quantitation was made by densitometric scanning of autoradiograms. Each figure represents the mean of two-four experiments. Error bars are indicated.

methylpsoralen, which in contrast to 8-methoxypsoralen predominantly gives rise to DNA monoadducts (results not shown), was also tested, and as shown in Figure 4b the synthesis of tRNA was slightly less sensitive than that of rRNA, 5S and 5.8S RNA at higher light doses.

In contrast to the above results, irradiation with short wavelength ulraviolet light (UVB) caused a preferential inhibition of rRNA, 5.8S and 5S RNA synthesis, while the synthesis of tRNA was nearly unaffected at the doses tested (Fig. 4c).

## DISCUSSION

In both pro- and eukaryotic cell systems it has been shown that the inhibition of RNA synthesis by UVB irradiation is most probably due to premature chain termination and release of the polymerase

**TABLE 1**

Ratio of 26S/17S of newly synthetized RNA after UVB irradiation.

| Irradiation (min) | 26S/17S ratio ± SE |
|---|---|
| 0 | 1.30 ± 0.1 |
| 0.5 | 1.65 ± 0.25 |
| 1 | 1.43 ± 0.15 |
| 2 | 1.25 ± 0.2 |

The RNA was analyzed as described in Figure 2 and the cells were treated as described in Figure 4c.

at the site of UV-damage (thymine dimer) (15-19). Consequently the synthesis of small RNA species is less sensitive to UVB radiation than that of large RNA species unless the small RNA's are an integrated part of a larger transcription unit (e.g. 5.8S RNA). Our finding that tRNA synthesis is much less sensitive to UVB-treatment than synthesis of rRNA, 5.8S and 5S RNA is therefore in accordance with previous findings. In contrast to the situation in E. coli (15), yeast (17) and mammalian cells (18-19), however, we find no change in the 17S/26S ratio of newly synthesized RNA even at UVB doses causing > 80% inhibition of RNA synthesis (Table I). This descrepancy could be due to the special pre-rRNA processing system in Tetrahymena involving a self-splicing intron (20), which is heavily dependent on the three-dimensional structure of the pre-rRNA. Therefore our result may indicate that correct rRNA processing, in vivo, in Tetrahymena requires an almost intact pre-rRNA and that prematurely terminated pre-rRNAs are degraded rather than processed.

The observation that the synthesis of tRNA is inhibited in parallel with that of rRNA, 5.8S and 5S RNA by 8-methoxypsoralen-UVA treatment is unexpected and has some interesting implications concerning both the molecular mechanisms for the inhibition of transcription by psoralen-DNA adducts as well as for the process of transcription itself.

The RNA transcripts analyzed in the present study can be divided into two groups, namely rRNA and 5.8S RNA which are members of the same transcription unit all being part of a pre-rRNA molecule (21) transcribed by polymerase I, and 5S and tRNA which are polymerase III transcription products. Furthermore, the transcrip-

tion of 5S RNA is probably effectively feed-back regulated by a 5S-binding transcription factor analogously to the case in Xenopus (22). Therefore the inhibition of 5S RNA synthesis may be due to trapping of the 5S-transcription factor by an initial surplus of 5S RNA, quickly arising due to inhibition of rRNA synthesis.

If the inhibition of RNA synthesis by psoralen-UVA treatment was primarily due to RNA chain termination at psoralen-DNA adducts the present results would indicate either that the transcription units of pre-rRNA (~ 6 kb) and those of the tRNA's were of equal size or that the part of the genome containing the tRNA genes were much more susceptible to psoralen-photoaddition. The first alternative seems extremely unlikely since the synthesis of tRNA is quite insensitive to UVB irradiation supporting the finding that tRNA primary transcripts are found to be short (~ 0.1 kb) (23). The second explanation is not plausible either, since a six hundred fold greater susceptibility for psoralen-adduct formation in the tRNA genes would be required, and no experiments to date even suggest that such great differences in terms of psoralen photoreactivity exist within the genome (2, 3, unpublished results). However, it cannot be totally excluded of course that the tRNA genes contain a "hot-spot" for psoralen addition. On the other hand it is highly unlikely that all tRNA genes would contain this "hot-spot" having exactly the observed susceptibility (~ 6 kb/0.1 kb ratio).

The present results could also be explained if the inhibition was due to an effect on the RNA polymerase itself rather than to the effect on the DNA template. It has been shown that psoralens by way of a photodynamic formation of singlet oxygen can cause inhibition of enzymes such as DNA polymerase (24), RNase (25) and ribosomes (26) in vitro. However, higher doses than the ones used in the present study of both light and psoralen are usually required in order to demonstrate the photodynamic effect. Furthermore, 3-carbethoxypsoralen (27,28) and also 3-carbethoxy-8-methylpsoralen (results not shown) are > 20 times as efficient as 8-methoxypsoralen in producing singlet oxygen. This relation is not reflected in the results concerning inhibition of RNA synthesis (Fig. 4a, b) since 8-methoxypsoralen is the more efficient of the two in this respect. Thus a photodynamic effect is not a likely explanation for the present results.

Finally the inhibition of RNA synthesis could be due to inhibition of initiation. This explanation has implications for the regulation of transcription itself. Assuming that the psoralen adducts are distributed evenly over the genome, the equal inhibition by 8-methoxypsoralen-UVA of tRNA and rRNA indicates that equally sized regulatory transcription domains exist for these genes, and that the size of these are 10-20 kb; the rDNA molecule having two transcription units constituting either one or two domains. It is noteworthy that this size is only slightly smaller than that (20-100 kb) suggested for chromosomal domains in higher eukaryotes (29).

The specific topoisomerase sites that have recently been found in the rDNA of Tetrahymena (30) as well as in the genome of higher eukaryotes (31) may also be relevant in this context, since both transcriptional and chromosomal domains may be topological domains (32), e.i., domains of a specific DNA superhelical density. It is not clear what feature of the psoralen DNA adducts are responsible for the inhibition of RNA synthesis. If, however, the mechanism is as proposed above, e.i., the turning off of entire transcriptional domains, DNA supercoiling may play a central role. It has been shown that psoralen-DNA interstrand crosslinks results in an unwinding of the DNA of ~ $28^{o}$ (33). Furhermore, we have obtained results indicating that psoralen-DNA interstrand crosslinks induce topoisomerase activity in Tetrahymena, probably as a step in the repair process (34). Thus the superhelical stress of entire domains may be released as a consequence of repair activity thereby turning off the activity of the promotors within the domains.

The analogous results obtained with 8-methoxypsoralen which forms both monoadducts and interstrand crosslinks and 3-carbethoxy-8-methylpsoralen which forms predominantly monoadducts (results not shown, 35) indicate that the domain mechanism may be operating with both types of adducts. It can be estimated, however, that crosslinks are approximately twenty times more potent than monoadducts since twice the concentration of 3-carbethoxy-8-methylpsoralen is required to produce an inhibition of RNA synthesis equal to that of 8-methoxypsoralen and it has been found that the relative quantum yield of 3-carbethoxypsoralen for formation of DNA adducts, in situ, is ~ 10 times that of 8-methoxypsoralen (35, 36, the presence of the 8-methyl group in the psoralen is not expected to have a drastic effect on the quantum yield (37, 38)).

The present results are at variance with the results of Nocentini who concluded that psoralen-DNA monoadducts produced by angelicin-UVA treatment resulted in inhibition of RNA synthesis in monkey kidney cells by way of premature chain termination (9). Whether this discrepancy is due to the different organisms or the different psoralens used remains to be seen.

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