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Differential inhibition of cellular RNAs by photosensitized trioxalen

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ABSTRACT

Treatment of mouse cell cultures with 5 x 10^{-6}M trioxalen (4,5',8-trimethylpsoralen, or TMP) followed by long wavelength ("near") UV-irradiation (NUV) resulted in immediate cessation of most of the cellular transcriptional activity. The effective inhibitory TMP concentrations for ribosomal RNA were lower than those affecting poly RNA containing RNA. Low molecular RNA species were only partially inhibited. Side effects, commonly caused by other drugs, were not detected. These properties, and the simplicity of their use, make psoralens attractive inhibitors of RNA biosynthesis.

INTRODUCTION

Psoralens, derivatives of furocoumarins, have been shown to easily penetrate cells and covalently cross-link cellular DNA strands upon irradiation with near UV light (1,2,3). The resulting inhibition of cellular mitotic activity makes them useful in the treatment of skin diseases such as psoriasis (4,5) and vitelligo (6). In recent years they were extensively used as probes for the structures of DNA, RNA and chromatin in cells and in viruses (for a review, see ref. 7).

Several psoralens were shown to inactivate cellular DNA as template for DNA and RNA biosynthesis (8,9,10,11). Although they can also photoreact with tRNA and rRNA, the extent of this reaction in intact cells is low (12) and, at least in TMP-NUV treated cells, does not interfere with translation of pre-existing messenger RNA (9,10). In this study we show that (i) mild treatment of cells with TMP-NUV, reported to introduce less than 7 cross-links per 1000 base pairs of cellular DNA (13) is sufficient to inhibit ribosomal and poly $(A)^+$ RNA synthesis; (ii) the inhibition of cellular RNA species is dependent on TMP concentration and (iii) devoid of detectable side effects.

MATERIALS AND METHODS

Cells. All experiments were conducted with the A9 derivative of mouse-L-cells (14). Some of the experiments were also repeated with Chinese hamster ovary (CHO) cells, with identical results. The cells were propagated in Dulbecco Modified Eagle medium supplemented with 5% fetal calf serum.

TMP-NUV treatment. Subconfluent cultures grown in 60-mm plates were washed once with phosphate-buffered saline (PBS). A drug solution in PBS (1 ml) was added, the plates were incubated at 37°C for 10 minutes and then irradiated at room temperature with two tubular fluorescent "black ray" lamps (UV products, Inc., San Gabriel, California, held in model XX-15 reflector). The lamps' maximal emission was about 360 nm, and the light intensity delivered to the samples was 2-4 mW/cm^2.

Radioactive labelling of RNA. Following TMP-NUV treatment, the cells were incubated in medium containing 5-20 µCi/ml of ^{3}H-uridine (Nuclear Research Center - Negev, 35 Ci/mmole). In the double-labelling experiments 2 µCi/ml of ^{14}C-uridine (New England Nuclear, 60 mCi/mmole) was added to the cells 12-24 hours prior to TMP treatment and washed away with PBS before the addition of the drug. After irradiation, ^{3}H-uridine-containing medium was added to the cells as described above.

Extraction of cytoplasmic RNA. Growth medium was removed, the plates were placed on ice and washed twice with 5 ml of cold PBS. The cells were scraped with a rubber policeman into 1 ml PBS, and collected by a brief (0.5 minute) centrifugation in an Eppendorf minicentrifuge. The cell pellet was suspended in 0.5 ml of hypotonic RSB solution (10 mM Tris-HCl pH 7.4, 10 mM KCl, 0.15 mM $MgCl_2$) and left in ice for 5 minutes. Nonidet P40 (Sigma) was added to the cell suspension to a final concentration of 0.5%, the mixture was then thoroughly vortexed and centrifuged for 1.5 minutes to remove nuclei and cell debris. The supernatant, containing the cytoplasmic fraction, was immediately removed to another tube and supplemented with SDS (0.5% final concentration) and EDTA (10 mM). An equal volume of a phenol-chloroform solution (2:1, v/v) was added, the mixture vortexed vigorously for 4-5 minutes, and centrifuged at room temperature to separate the phases. The aqueous phase was similarly re-extracted twice more, and the RNA was finally precipitated with 2.2 volumes of ethanol and 0.1 M NaCl at -20°C.

Velocity sedimentation analysis of RNA. The labelled RNA samples were dissolved in 75% dimethyl sulfoxide (DMSO), heated at 60°C for 3 minutes, and placed on top of a 15-30% (w/w) sucrose gradient in 0.01 M Tris-HCl pH 7.0, 0.1 M NaCl, and 0.01 M EDTA. Gradients were spun in a SW 50.1 rotor

at 45,000 rpm for 5.5 hours, 4°C. Fractions were collected, their RNA content was precipitated by cold, 5% TCA, filtered through glass-fiber filters (GF/C, Whatman), and counted in toluene-based scintillation fluid in a Packard scintillation counter in single or double labelling setting according to the radioactive label used.

Oligo(dT)-cellulose fractionation was done as previously described (15).

Polyacrylamide gel electrophoresis of RNA. The RNA samples were dissolved in 75% DMSO, heated at 60°C for 3 minutes and applied to 10% polyacrylamide cylindrical gels. Electrophoresis was at 6 mA/gel for 3.5 hours in TBE buffer (0.04 M Tris pH 8.3, 0.45 M boric acid, 0.0014 M EDTA). The gels were then sliced by a gel slicer. Each 2 mm slice was extracted by shaking overnight in 0.5 ml of 0.1 x NaCl/Cit (1 x NaCl/Cit is 0.15 M NaCl, 0.015 M trisodium citrate). Dioxane-based scintillation fluid was added and samples were counted in a double labelling setting.

RESULTS

1. Effect of irradiation dose and TMP concentration on transcription.

For optimal inhibition of RNA biosynthesis, TMP was added to the cultures at least 5 minutes before irradiation. Longer preincubation did not have any effect on the consequent extent of inhibition. In general, the drug alone did not seem to affect cellular metabolism: cells propagated in the presence of 5×10^{-6} M TMP (its maximal solubility in PBS) for over a week divided at a normal rate with no apparent cytotoxic effects. Their plating efficiency in the presence of the drug was identical to that measured in normal medium.

Figure 1 illustrates the effect of irradiation from a near UV source on cells which were pre-treated with various TMP concentrations. The effective inhibitory concentrations for both DNA and RNA synthesis were in the range of 5×10^{-9} M and 10^{-7} M. Complete inhibition, however, could not be achieved: a residual synthesis amounting to 10-13 percent of control cells (untreated or treated with TMP or NUV alone) for RNA, and 2-3 percent for DNA was obtained in cells treated with 5×10^{-6} M TMP.

The dose response for inhibition of RNA synthesis was studied by treating the cells with 5×10^{-6} M TMP and irradiating them for various time periods (fig. 2). The residual synthesis could not be eliminated or significantly reduced using irradiation periods longer than 10 minutes, but repeated irradiations, each with a fresh TMP solution, reduced it considerably.

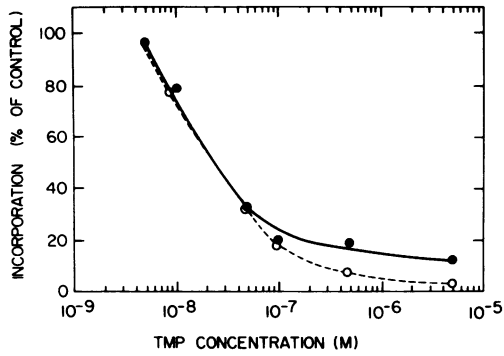

FIGURE 1. Effect of TMP concentration on DNA and RNA biosynthesis. A9 cells were grown in monolayer in medium containing ^{14}C-uridine for 24 hours. The plates were treated with different concentrations of 4,5',8-trimethylpsoralen, irradiated at room temperature for 5 minutes and labelled with ^{3}H-uridine as described in materials and methods. Another set of plates was similarly labelled with 2μ Ci/ml of ^{3}H-thymidine. Cells lysis and counting was done as described in the legend to figure 1. The ^{3}H counts in each sample were normalized to a standard number of ^{14}C counts, and the inhibition was calculated as percentage of untreated cells. ●, ^{3}H-uridine; o, ^{3}H-thymidine.

2. Differential inhibition of ribosmal RNA and of poly(A)$^{+}$ RNA by TMP-NUV.

The wide range of TMP concentrations over which inhibition took place (figure 1) suggested that the drug was a differential inhibitor of cellular RNA species. To test this, cytoplasmic RNA from cells treated with various

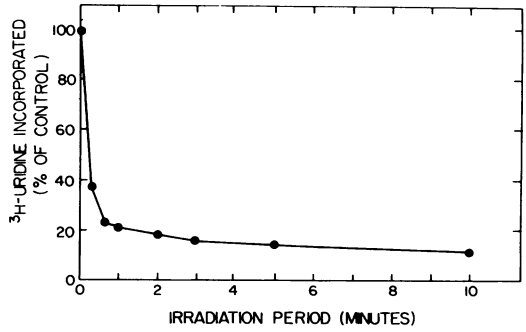

FIGURE 2. RNA synthesis as function of irradiation time. Plates containing identical numbers of cells (1.5 x 10^{6}) were treated with TMP and irradiated with NUV as described in materials and methods. After labelling for 4 hours with ^{3}H-uridine, the cells were washed and collected in cold PBS, centrifuged, and lysed in 0.5 ml of a solution containing 10 mM Tris pH 7.4. 150 nM NaCl, 3 mM $MgCl_2$, 0.25 M sucrose, and 0.5% Triton X-100. The nucleic acids were precipitated in 5% TCA, filtered and counted.

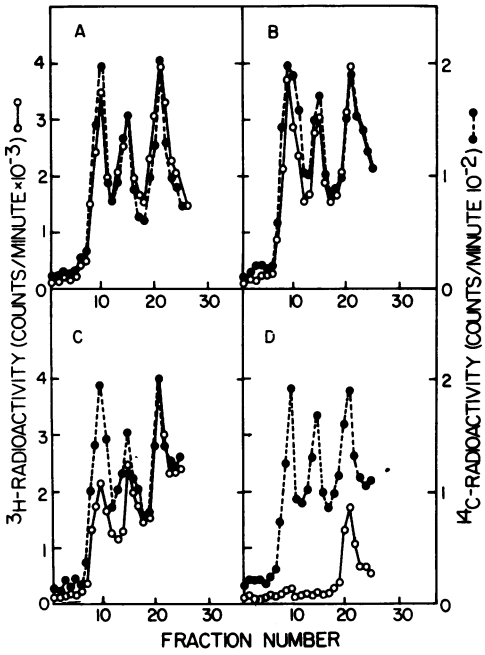

FIGURE 3. Inhibition of ribosomal RNA synthesis by TMP-NUV. Cells were labelled to a steady state (16 hours) with ^{14}C-uridine, treated with TMP-NUV and labelled for 4 hours with ^{3}H-uridine. RNA was extracted and subjected to velocity sedimentation analysis on sucrose gradients as detailed in Materials and Methods. (A) Control, NUV irradiated; (B) 10^{-8} M TMP, NUV irradiated; (C) = 2.5×10^{-8} M TMP, NUV irradiated; (D) 5×10^{-6} M TMP, NUV irradiated.

TMP concentrations and followed by NUV irradiation, was analyzed by velocity sedimentation in sucrose gradients. The double labelling technique was used in this experiment: cells were labelled to a steady-state with ^{14}C-uridine prior to TMP-NUV treatment, and ^{3}H-uridine was used to label the residually synthesized RNA, thus providing an internal standard of normal cellular RNA. Some of the sedimentation profiles obtained are presented in figure 3. The results show that (i) the bulk of ribosomal RNA synthesis was inhibited over a narrow range of TMP concentrations (10^{-8} M to 5×10^{-8} M); (ii) the synthesis of low molecular weight RNA was only partially inhibited (about 50 percent) at maximal TMP concentration; and (iii) no apparent degradation of pre-existing ribosomal RNA took place at any of the drug concentrations used. The percentage of the poly A containing fraction in unfractionated pre-existing RNA, 4 hours after irradiation, was determined by oligo(dT)-cellulose chromatography and found to be similar to that of untreated control cells (2.4 - 2.6%). Furthermore, if selective degradation of poly $(A)^+$ RNA took place, one would expect that after size fractionation, an increased fraction of low molecular weight RNA would bind to oligo(dT)-cellulose. The results of this analysis (not shown) did not reveal any increase of the poly $(A)^+$ RNA fraction in the 4S-6S size region of both ^{14}C-labelled (pre-existing),

TABLE 1. Synthesis of total and of poly $(A)^+$ RNA in TMP-NUV and actinomycin D treated cells

Treatment of cells	^3H uridine incorporation (%)	% poly $(A)^+$ RNA
None or 5×10^{-6} M TMP	100[a]	1.9
NUV	96	2.1
Act. D[b]	48	8.7
TMP-NUV[c]	12.1	2.7
TMP-NUV + Act. D[d]	9.8	2.9
TMP-NUV + Act. D[e]	7.8	2.5

TMP-NUV treatment and labelling conditions were described in Materials and Methods. The results represent the average of 4 separate experiments. (a) 399,000 cpm. (b) 0.04 µg/ml. (c) 5×10^{-6} M TMP treatment followed by irradiation as in figure 1. (d) Cells were treated with TMP-NUV as in (c) and incubated for 20 minutes in medium containing actinomycin D (0.04 µg/ml) before the addition of ^3H uridine. (e) 5×10^{-6} M TMP; 0.5 µg/ml actinomycin D.

and ^3H-labelled (post-treatment) RNAs, compared to that of the control RNA.

Evidence that TMP-NUV treatment inhibits the synthesis of poly A containing RNA is presented in Table 1. Exposure of cells to a low dose of actinomycin D, known to preferentially inhibit ribosomal RNA synthesis, resulted in an elevated poly $(A)^+$ RNA fraction, from about 2% in the control cells to around 9%. The combined treatment of low actinomycin D with TMP-NUV yielded close-to-normal percentage of the poly $(A)^+$ fraction. The combined treatment of high dose actinomycin D with TMP-NUV slightly reduced the overall level of residual synthesis, but the poly $(A)^+$ fraction of this RNA was similar to that obtained with low actinomycin D + TMP-NUV or with high actinomycin D alone. These results indicate that TMP-NUV is roughly as efficient as high actinomycin D in the inhibition of poly $(A)^+$ RNA synthesis.

Figure 4 demonstrates the differential inhibitory effect of TMP-NUV on ribosomal RNA and poly $(A)^+$ RNA. Two series of cultures containing identical numbers of cells were treated with different TMP concentrations. One set of plates was also treated with a low dose of actonomycin D so that incorporation of uridine in these plates was primarily into non-ribosomal RNA. The solid curves, representing the total residual synthesis, show that while inhibition of RNA synthesis in the absence of actinomycin D started at 5×10^{-9} M TMP (upper panel), no further inhibition of actinomycin D residual synthesis took place below 2×10^{-8} M TMP (lower panel), indicating that low TMP

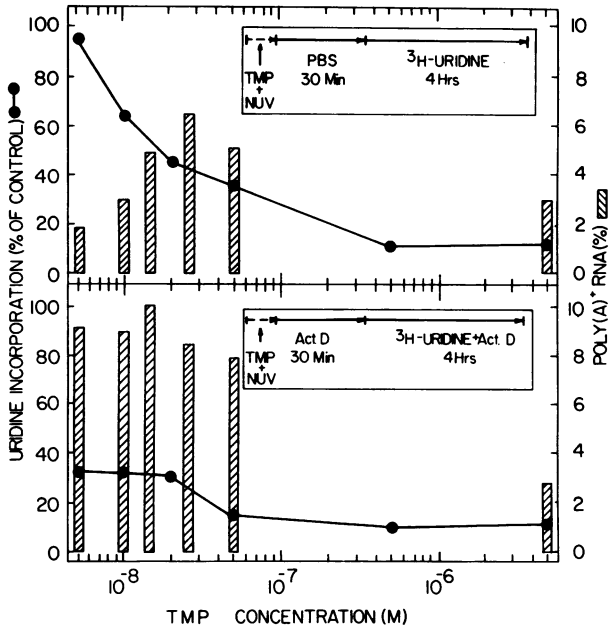

FIGURE 4. Inhibition of poly $(A)^+$ RNA synthesis by TMP-NUV. Cells were treated with various concentrations of TMP, irradiated with NUV for 5 minutes, and treated with ^{3}H uridine in the absence (top) or presence (bottom) of actinomycin D (0.04 µg/ml). Cells were collected by centrifugation, suspended in PBS and samples were taken for direct counting of uridine incorporation as in figure 1. The RNA was extracted from the rest of the cells, and the percentage of poly $(A)^+$-containing RNA in each sample was determined (15). For details, see Materials and Methods and the experimental protocols in the inserts.

concentrations affected primarily RNA species which were also inhibited by low actinomycin D.

We also determined the percentage of poly $(A)^+$ RNA in the residually synthesized cytoplasmic RNA of each TMP concentration using oligo(dT)-cellulose chromatography. The results of this analysis are superimposed on the synthesis curves in figure 4 in a histogram form. The data in the upper panel show that, concomitantly with a progressive decrease in ribosomal RNA synthesis by increasing TMP concentrations, there was an increase in the relative fraction of poly $(A)^+$ RNA from about 2% at 5×10^{-9} M TMP to a maximum of 6% at 2.5×10^{-8} M. At higher TMP concentrations there was a gradual decrease in the poly $(A)^+$ RNA fraction, reaching 3.1% at 5×10^{-6} M. In comparison, the poly $(A)^+$ RNA percentage of actinomycin D treated cells

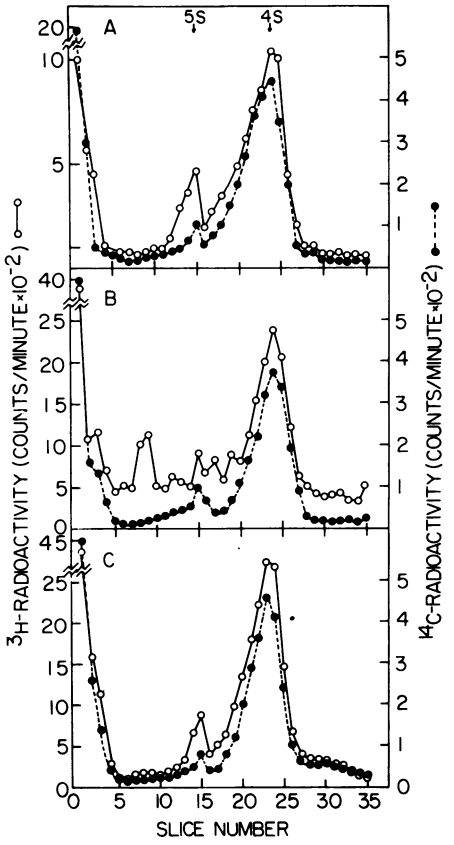

FIGURE 5. Polyacrylamide electrophoresis of TMP-NUV and Act. D RNAs.
RNA from cells treated with TMP-NUV and from control cells was prepared as in figure 2. Act. D RNA was prepared as follows: after ^{14}C-uridine labelling, the cells were washed twice with PBS, and supplemented with a medium containing 5 $\mu g/ml^{-1}$ actinomycin D, incubated for 20 minutes at 37°C. ^{3}H-uridine was added, and 4 hours later cytoplasmic RNA was extracted.

(lower panel) was substantially higher (9-10%) and was unaffected by treatments with low TMP concentrations followed by NUV irradiation. We conclude that TMP-NUV is a differential inhibitor of ribosomal and poly $(A)^+$ RNAs. Its discriminatory effect, however, is not as distinct as that of actinomycin D, since the enrichment in the poly $(A)^+$ fraction was only 6% as compared with 9% obtained with actinomycin D (Table 1).

3. Residually synthesized RNA contains mainly low molecular weight RNA.

Since actinomycin was reported to allow the synthesis of some low molecular weight RNA (16,17,18) it was of interest to compare it to the RNA synthesized in TMP-NUV treated cells. The double labelling technique was again used, and the RNAs from cells treated with each drug were subjected to polyacrylamide gel electrophoresis. Panel A in figure 5 shows that the predominant species of TMP-NUV RNA were 4S and 5S. Clearly, no degradation

products of pre-existing, ^{14}C-labelled RNA were detected. RNA greater than 8S was confined to the origin of the gel and amounted to about 50 percent of the label in the entire gel. When the cells were subjected to four consecutive TMP-NUV treatments, each one with a fresh drug solution, lower relative amounts of 4S and 5S RNAs were synthesized, but the RNA at the origin of the gel constituted only 15 percent of the total label. Thus, repeated treatments affected primarily the synthesis of high molecular weight RNA species. Compared to TMP-NUV RNA, actinomycin D RNA was less homogeneous in size (panel B): beside 4S and possible 5S species, a number of low molecular weight RNA species larger than 5S were separated. These are likely to be degradation products which leaked from the nuclei during cell fractionation.

It has been previously shown that the low molecular weight RNA synthesized in actinomycin treated cells was largely ribonuclease resistant, possibly double stranded (17,18). We have isolated from sucrose gradients the 4S RNA from TMP-NUV treated cells, as well as from untreated control cells, and subjected them to ribonuclease digestion under various salt conditions. We could not find any increased resistance of the TMP-NUV RNA to nuclease digestion over that of the control. Furthermore, the isolated RNAs were subjected to digestion with the single strand specific S1 nuclease which has been shown to cleave tRNA in its anticodon region (19,20). The cleaved RNAs were then subjected to electrophoresis on a 16% polyacrylamide gel (19). The mobility of both RNA samples was faster than that of tRNA, and corresponded to that of fragments 30 to 50 nucleotides long (data not shown). These results suggest that the main low molecular weight species in the residual RNA synthesis is transfer RNA, but a direct identification by aminoacylation was not done.

4. The effect of TMP-NUV treatment of cell viability.

The metabolic viability of the treated cells was examined by studying the kinetics of uridine incorporation at different times after irradiation (Figure 6). The results show that immediately after the treatment, the rate of RNA synthesis has been reduced 6-fold. This rate remained relatively stable for at least 6 hours and was further reduced by 9 hours after treatment. The 6-fold reduction reflected the inhibition of ribosomal and of poly $(A)^+$ RNAs in the treated cells. It is likely that the further rate decrease at 9 hours reflects a deterioration in the physiological state of the treated cells. We examined the cells microscopically over a period of 20 hours after TMP-NUV treatment and determined the percentage of dead cells by counting them in the presence of trypan blue. At 6 hours after treatment no dead

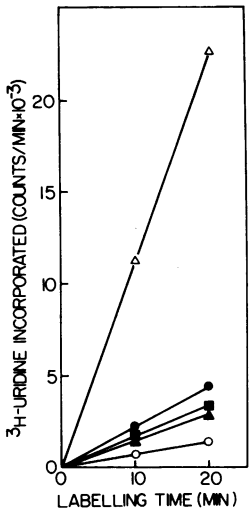

FIGURE 6. Transcriptional activity of TMP-NUV treated cells.
Cultures were pulse labelled with ^{3}H-uridine at intervals following TMP-NUV treatment, and incorporation into RNA was measured by TCA precipitation. △ Control, NUV-treated cells; ●, time zero; ▲, 3 hours post treatment; ■, 6 hours post treatment; ○, 9 hours post treatment.

cells were detected. Nine and twenty hours after treatment, dead cells constituted 15% and 25%, respectively.

DISCUSSION

The commonly used inhibitors of transcription suffer from two major drawbacks. Firstly, an obligatory time lag takes place from the moment of the addition of the drug until it reaches the effective inhibitory concentration in the cell nucleus. The use of psoralens overcomes this problem: since the unirradiated drug is inactive, it can be added to the cells in advance and inhibition can then be affected by irradiation whenever desired.

Secondly, a number of side effects associated with the use of drugs have been described in the literature. We assume that at least some of these undesirable effects are caused by the continued presence of the active form of the drug during the entire inhibition period, and that they can be reduced, or even eliminated by the use of psoralens whose mode of action is transitory and irreversible. We have searched in TMP-NUV treated cells for some of the side effects which were observed during the use of other drugs, notably actinomycin D. There have been reports that, at high actinomycin D concentration, a breakdown of polyribosomes takes place, probably as a result of an interference of the drug with the initiation of translation (21,22). We failed to observe polysome breakdown in our systems for at least 4 hours after TMP-NUV treatment (data not shown).

Actinomycin D has been also reported to cause artificial breakdown of RNA in cells (16). Our studies failed to observe accelerated degradation of ribosomal and of poly (A) containing RNAs until 4 hours following TMP-NUV treatment. Although these findings seem to contradict those of Fredriksen and Hearst, who found increased RNase activity and pronounced breakdown of RNA after treatment (12), one has to take into consideration that a highly soluble trioxalen derivative (4'-aminomethyl trioxalen) was used by this group, and that it was applied to the cells at a molar concentration 100-fold higher than that used in our experiments with TMP. Furthermore, Heimer et al. showed that TMP-NUV treatment of CHO cells did not interfere with the translation of pre-existing messenger RNA (9,10). That the biological activity of nucleic acids is affected more than that of proteins by these drugs was demonstrated in psoralen-treated NUV-irradiated Rous sarcoma virus: although DNA synthesis in the treated virions was decreased, virion DNA polymerase, assayed with exogenous RNA template, was not significantly altered (23).

Our results revealed a close similarity in the inhibitory effect of actinomycin D and TMP-NUV: both are differential inhibitors of poly $(A)^+$ and ribosomal RNAs, and low molecular weight RNA partially escapes inhibition by both drugs. They seem, however, to inhibit different steps of the transcription process: TMP-NUV inhibition appears to be an "all or none" effect, suggesting interference with the initiation stage of transcription (24). In contrast, actinomycin D was shown to inhibit RNA chain elongation (25,26) giving rise to short, premature termination products of RNA (27). In our experiments, short transcripts appeared in actinomycin D RNA and not in TMP-NUV RNA or control RNA (Figure 5). Their association with the cytoplasmic fraction, however, suggests that they result from nuclear leakage rather than being premature termination products of transcription. Their preferential presence in actinomycin D RNA is a matter for further investigation.

Although psoralen derivatives with higher solubility are now available (28), the use of TMP seems to be advantageous for transcription-inhibition studies: firstly, its photoreaction with DNA is initially 3-fold faster than that of the highly soluble AMT, thus enabling shorter irradiation periods (13); secondly, photoreaction of TMP with purified DNA was shown to yield a maximum of 24.4 bound drug molecules per 10^3 base pairs, and 28% of that with isolated chromatin (13). In the experiments reported here, whole cells were used and, in addition, the irradiation intensity applied to the cells

was 2-5 fold lower. We therefore assume that under these conditions less than 7 crosslinks per 1000 base pairs of DNA were formed. While the inhibition caused by this low level of binding is as efficient as that of 0.5 μg/ml actinomycin D (Table 1) it ensures also low level binding of the drug to cellular RNAs and proteins, and as a consequence minimizes potential, undesirable side effects.

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