Effect of lucanthone (miracil D) on transcription of ribosomal RNA genes from Tetrahymena
in vivo and in vitro

Ole Westergaard, Elmar Gocke, Ole F. Nielsen and Johan C. Leer

Department of Molecular Biology and Plant Physiology, University of Aarhus, DK-8000 Aarhus C, Denmark

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ABSTRACT

Addition of lucanthone (1-5 μg/ml) to cultures of Tetrahymena results in a preferential inhibition of the synthesis of ribosomal RNA. Transcriptional studies with isolated nucleoli from Tetrahymena demonstrate that the endogenous RNA polymerases of the r-chromatin (chromatin form of rDNA) do not recognize the normal termination and move into the spacer region distal to the terminator in the presence of lucanthone. This is shown by hybridization of the transcript synthesized in the presence of the drug to restriction fragments of rDNA. Lucanthone seems specific in its action on termination as it does not inhibit the elongation process on the chromatin. Among various DNA-binding drugs tested only lucanthone and proflavine are found to cause repression of the termination. The data obtained suggest that the reduced synthesis of rRNA in lucanthone-treated eukaryotic cells is due to lack of reinitiating RNA polymerases possibly caused by improper termination.

INTRODUCTION

Lucanthone is a drug with a heteroaromatic ring-structure which resembles that of actinomycin D. It interacts with nucleic acids and is a potent inhibitor of RNA synthesis in both prokaryotes and eukaryotes. At low concentration the drug selectively inhibits the synthesis of ribosomal RNA in Hela cells. We have recently developed methods which makes it possible to isolate highly purified r-chromatin and nucleoli from exponentially growing cultures of Tetrahymena. The isolated nucleoli contain transcriptionally active r-chromatin; the transcriptional properties of the system mimic the in vivo process with respect to (i) strand selection, (ii) elongation rate and (iii) termination. The current studies were undertaken to determine whether lucanthone had a specific effect on any of
these processes. The presented results demonstrate that the drug at 10% concentration has a specific effect on the termination process, while we observe no effect on strand selection and elongation rate. We compare these results with the in vivo effect of lucanthone and propose a model for the action of the drug in vivo.

MATERIALS AND METHODS

Preparation of nuclei. Cultures of Tetrahymena pyriformis, strain GL (micronucleate) were grown to a density of 60-80,000 cells/ml. Cells treated for 30 min with lucanthone or untreated cells were harvested by centrifugation at 300xg and washed with 30 volumes of nuclei buffer (0.1 M sucrose, 10 mM Tris pH 7.2, 3 mM CaCl₂, and 10 mM NaCl) at 4°C. The nuclei were prepared by lysis of cells in 30 volumes of nuclei buffer containing 0.3% Nonidet P40. They were collected by centrifugation at 500xg, washed once with 30 volumes of nuclei buffer containing 0.1% Nonidet P40 and once with 30 volumes of nuclei buffer. The nuclei were resuspended in 5 volumes of extraction buffer (10 mM Tris-HCl pH 7.2, 140 mM NaCl, 1 mM MgCl₂, 1 mM 2-mercaptoethanol and 10% glycerol).

Preparation of nucleoli. Nucleoli were prepared from the isolated nuclei after resuspension in 10 volumes of extraction buffer at 4°C and exposure to gentle homogenization in a glass homogenizer. The extracted nuclei were removed by centrifugation at 500xg and the nucleoli in the supernatant layered on top of a 4.5 ml sucrose cushion (3.0 ml of 50% sucrose and 1.5 ml of 80% sucrose in 10 mM Tris pH 7.2, 140 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol) in a SW27 centrifuge tube. After 30 min of centrifugation at 15,000xg, nucleoli were collected in the bottom 3 ml of the cushion. The nucleoli contain r-chromatin corresponding to 8-16 µg rDNA/ml in a purity of 90-95%.

Determination of RNA polymerase activity. The endogenous RNA-polymerase activity on the r-chromatin was determined by incubation with an assay mixture containing 4.9 mM (NH₄)₂SO₄, 8.2 mM MgCl₂, 3.8 mM 2-mercaptoethanol, 0.50 mM EDTA, 32 mM Tris-HCl pH 7.2, 2.1 mM KCl, 57 mM NaCl, 95 µM of ATP, GTP, and CTP, and 7.5 µM of 5,6-³H-labelled UTP (2 Ci/mnmole). All in-
cubations were performed with an amount of r-chromatin corresponding to 4-8 μg rDNA/ml. After incubation at 25°C for 10 min, acid-insoluble counts in 75 μl were determined as described. One unit of RNA-polymerase activity is defined as the amount of enzyme which incorporates one pmole of UMP into RNA in 10 min at 25°C. In different preparations the RNA-polymerase activity varied from 600-1800 Units per μg rDNA.

RNA-size and elongation-rate studies. For gel studies an incubation mixture similar to the above mentioned was used except that 30 μM α<sup>32</sup>P-labelled UTP (2 Ci/mmmole) was used. RNA-size studies were done on 1.8% agarose gels containing 5 M urea by the method of Dudov et al. Control studies on formamide gels gave results identical to those on the urea gels. The gels were calibrated with marker RNA from EMC (molw. 2.5 x 10<sup>6</sup> daltons), TMV (molw. 2.0 x 10<sup>6</sup> daltons), and ribosomal RNAs from rat liver (18S and 28S) and Eschericia coli (16S and 23S). A semilogarithmic plot of the weight versus the migration in the gel gave a straight line. All elongation-rate studies were performed with nucleoli stripped for the in vivo synthesized RNA chains and the length of the in vitro synthesized chains were estimated from the gels as described in ref. 7 and 8.

Preparation of DNA complementary to 17S and 25S rRNA. Tetrahymena ribosomes were dissociated with EDTA and 30S and 50S subunits separated in sucrose gradients. Peak fractions were extracted with phenol-chloroform and after alcohol precipitation the 17S and 25S rRNA was sedimented in sucrose gradients containing SDS. The 17S and 25S rRNA collected from these gradients were precipitated with alcohol. cDNA was synthesized from 17S and 25S rRNA after polyadenylation according to the method of Hell et al. cDNA of 3-6S was collected from alkaline gradients. Backtitration with 17S and 25S rDNA has shown that all sequences in the 17S and 25S rRNA are equally represented in the complementary DNA (data not shown). Cross contaminations of 1.5% and 6% were found in the cDNA of 17S and 25S rRNA, respectively.

Hybridization. In vitro transcription product for hybridization was synthesized in the nuclear preparations under standard assay conditions except that the ATP concentration was 15 μM (4 Ci/mmmole of α<sup>32</sup>P-ATP) and CTP and Hg-CTP were present in con-
centrations of 15 μM each. This reduction in concentrations of nucleotides has no effect on the size of the product as $K_M$ for the enzyme is determined to approximately 5 μM. Time of incubation was 15 min. The transcript was cleaved at pH 11 to an average size of 800 nucleotides and purified on Thiol Sepharose 4B (Pharmacia). Fixed amounts of transcript were titrated with cDNA to 17S and 25S rRNA, separately and combined. The samples were incubated to a Rot of 0.7 mole · liter$^{-1}$ · sec$^{-1}$, which is sufficient to drive more than 95% of the complementary sequences into hybrid (the kinetic complexity of the precursor rRNA corresponds to a Rot of $2.3 \cdot 10^{-2}$ moles · liter$^{-1}$ · sec$^{-1}$). Hybridizations and assays of hybrid resistant to S1 nuclease were done according to the method of Hell et al. 10.

The amounts of cDNA and transcript were calculated from the specific activity of $^3$H-dCTP and $^{32}$P-ATP assuming equal representation of all four bases in cDNA and transcript. For control hybridization Tetrahymena ribosomes were labelled in vivo with $^{32}$P-orthophosphate (specific activity ≈ 300.00 cpm/μg). The ribosomes were extracted with phenol-chloroform and total 17S + 25S rRNA collected from SDS-sucrose gradients without prior separation of subunits. The rRNA was niched as above and challenged with cDNA to 17S and 25S rRNA in order to get estimates of the background caused by secondary structure, and maximal efficiency of the hybridization.

The transcript hybridized to restriction fragments of rDNA was synthetized in isolated nucleoli using the incubation mixture described above. The transcript was purified on Thiol Sepharose 4B columns (Pharmacia) 11 and hybridized to fragments of rDNA generated by digestion with restriction endo-nuclease Bam H-1 (New England Biolabs), separated on 1.2% agarose gels, and transferred to cellulose nitrate filters 12. The rDNA used for endonuclease digestion was extracted from isolated nucleoli and centrifuged to equilibrium in a CsCl-Hoechst 33258 gradient. Gel analyses have shown that the DNA is more than 95% pure.

Chemicals. Lukanthone, hycanthone, and proflavine were gifts from Sterling-Winthrop Research Institute (Rensselaer, New York), ethidium bromide was purchased from Sigma (St. Louis, Mo.), and actinomycin D from PL-Biochemical (Milwaukee, Wisc.).
All isotopes were obtained from The Radiochemical Centre (Amer-sham, England).

RESULTS

Previously it has been shown that exposure of Hela cells to low concentrations of lucanthone (2-5 μg/ml) causes a 50-80 fold reduction in the synthesis of ribosomal RNA, while the synthesis of heterogeneous RNA is only slightly inhibited. Thus lucanthone has a selective effect on the expression of ribosomal genes.

In order to obtain more detailed information about the interaction of lucanthone with transcription of ribosomal RNA genes, we have by hybridization experiments studied the synthesis of ribosomal RNA in nuclei from cells treated with the drug in vivo. This is possible as the in vitro-synthesized RNA can be separated from the in vivo product after incorporation of the 5'-mercury derivative CMP into RNA during transcription in the isolated nuclei.

In nuclei from cells treated with lucanthone there is a general reduction in the total RNA synthesis. For instance at a concentration of 1 μg/ml the synthesis is approximately 50% of the control level. Like in Hela cells the drug has a preferential effect on the expression of ribosomal RNA genes. This can be seen from Table 1, where the synthesis of rRNA is given in

Table 1. Synthesis of ribosomal RNA in isolated nuclei from cells treated with varying concentrations of lucanthone

<table>
<thead>
<tr>
<th>Concentration of lucanthone in growth medium (μg/ml)</th>
<th>0</th>
<th>0.2</th>
<th>1.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of rRNA relative to total RNA synthesized</td>
<td>56%</td>
<td>45%</td>
<td>38%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Nuclei were isolated from cells treated with varying concentrations of lucanthone. RNA was synthesized in the presence of Hg-CTP and purified on Thiol Sepharose 4B columns. Fixed amounts of the in vitro synthesized transcript was titrated with cDNA to 17S and 25S rRNA. (For details see Methods.)
per cent of total RNA synthesized after treatment of cells with varying concentrations of lucanthone.

As it is possible to isolate transcriptionally active nucleoli\(^6,\,^7\), the effect of lucanthone on the transcription of ribosomal RNA-genes can be studied on a molecular level. The transcription of isolated nucleoli mimics the \textit{in vivo} transcription in several respects. Thus, the \textit{in vitro} transcription results in synthesis of a \(2.3 \times 10^6\) daltons transcript identical to the largest stable \textit{in vivo}-synthesized precursor (Figure 1, lane 1). Exposure of the isolated nucleoli to low concentrations of lucanthone results in synthesis of a certain percentage of transcripts with a molecular weight of \(3.0 \times 10^6\) daltons indicating that the normally non-transcribed spacer region distal to the termination signal is transcribed. Figure 1 (lanes 2–7) shows the increased size of the transcript after addition of lucanthone in concentrations up to \(240 \mu g/ml\). In the presence of \(180 \mu g/ml\) (Figure 1, lane 6), approximately one fifth of the polymers

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Effect of lucanthone on the size of the synthesized transcript}
\end{figure}

Samples of nucleoli were incubated for 30 min with \(\text{\textsuperscript{32}P}\)-labelled assay mixture in the presence of lucanthone: (1) 0 \(\mu g/ml\); (2) 15 \(\mu g/ml\); (3) 30 \(\mu g/ml\); (4) 60 \(\mu g/ml\); (5) 120 \(\mu g/ml\); (6) 180 \(\mu g/ml\); (7) 240 \(\mu g/ml\). All samples were deproteinized and applied on 1.8% agarose gels with 5 M urea.
Rase molecules have moved into the distal spacer region. At a concentration of 240 μg/ml, an inhibition of the total rRNA synthesis is observed (Figure 1, lane 7).

The restriction enzyme Bam H-1 has three cleavage sites within the rDNA molecule\textsuperscript{13,14} (Figure 2A), two of which are localized in the spacer region distal to the normal termination point. Figure 2B shows that rRNA synthesized in nucleoli in the absence of lucanthone hybridizes only to the two fragments localized in the normally transcribed region (coding for 17S and 25S rRNA plus transcribed spacer). However, rRNA synthesized in the presence of lucanthone (120 μg/ml) hybridizes to all four fragments. This shows that the endogenous RNA-polymerase molecules on the r-chromatin in the presence of lucanthone read

![Restriction Map and Hybridization](image)

**Figure 2.** A. Bam H-1 restriction map of the extra-chromosomal, palindromic rDNA from Tetrahymena (cf. ref. 13, 14).

B. Hybridization of transcripts synthesized in the absence or presence of lucanthone to Bam H-1 restriction fragments of rDNA.

Transcripts were synthesized in isolated nucleoli and purified as described in Methods: (1) in the absence of lucanthone; (2) in the presence of 120 μg/lucanthone/ml. Each lane represents the digest of 0.5 μg rDNA. Hybridization to excess amounts of transcript was done according to the method of Southern\textsuperscript{12}. 
through the normal termination point. As the transcript synthesized in the presence of the drug hybridizes to both of the two distal restriction fragments, the endogenous RNA polymerases must transcribe the spacer region to the end or near to the end of the rDNA molecule.

Lucanthone seems to interact specifically with the termination process as we observe no effect on the elongation rate of transcription. The elongation rate was determined on nucleoli stripped for in vivo-synthesized RNA chains. The time course of RNA-chain growth was followed after incubation of the stripped nucleoli in 32P-labelled assay mixture. At different times of incubation, the reaction was stopped and the size of the transcript measured on agarose-urea gels (Figure 3). The rates calculated from these gel studies are given in Table 2. Within the first eight minutes of incubation, the rate is nearly constant.

![Figure 3](image)

**Figure 3.** Elongation rates of endogenous RNA polymerase (A) in the absence and (B) presence of lucanthone (120 μg/ml)

Isolated nucleoli were stripped for the in vivo-synthesized RNA and incubated with 32P-labelled assay mixture. Aliquots were taken after the following times of incubation: (1) 2 min; (2) 4 min; (3) 6 min; (4) 8 min; and (5) 12 min. All samples were deproteinized and applied on a 1.8% agarose gel with 5 M urea.
Table 2. Elongation rates of the endogenous RNA polymerase on r-chromatin

<table>
<thead>
<tr>
<th>Incubation periods (min)</th>
<th>Elongation rate (nucleotides/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>2-4</td>
<td>7.9</td>
</tr>
<tr>
<td>4-6</td>
<td>8.3</td>
</tr>
<tr>
<td>6-8</td>
<td>7.6</td>
</tr>
<tr>
<td>8-12</td>
<td>3.4</td>
</tr>
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</table>

The experiment was performed as described in Methods. Elongation rates are calculated based on the positions of the front of the spots in Figure 3.

stant and independent upon the presence of lucanthone. After 8-12 min the elongation rate in the presence of lucanthone is twice as high as in the control. During this period the polymerases are approaching the normal termination point, a fact which might explain the difference in the observed elongation rates.

Recently, we have reported that addition of salt allows the endogenous RNA polymerase partly to read through the normal termination point\(^6\) (see also Figure 4, lane 2). Figure 4 shows a cooperative effect of salt and lucanthone. Thus, 25 μg/ml of lucanthone is sufficient to delete termination completely in the presence of 125 mM of ammonium sulfate (Figure 4, lane 3) compared to a suppression of termination of <5% in the presence of lucanthone alone (30 μg/ml, cf. Figure 1, lane 3) and 50% in the presence of ammonium sulfate alone (125 mM, cf. Figure 4, lane 2).

Lucanthone shares the tricyclic heteroaromatic ring system with a number of other components such as quinacrine, hycanthone, proflavine, actinomycin D, and acridine orange. All interact with nucleic acids and were therefore tested in concentrations ranging from 0.2-200 μg/ml for their effect on the termination. Besides lucanthone only proflavine was observed to suppress normal termination. The optimal effect of proflavine was found at a concentration of about 40 μg/ml. As for lucanthone, proflavine did not inhibit the elongation process. Hycanthone is a product in one of the metabolic pathways for lucanthone.
Figure 4. Effect of ammonium sulfate and lucanthone on the size of the synthesized transcript

Samples of nucleoli were incubated for 30 min with $^{32}$P-labelled assay mixture in the presence of salt and/or lucanthone: (1) control; (2) 125 mM ammonium sulfate; (3) 125 mM ammonium sulfate + 25 μg lucanthone/ml; (4) 125 mM ammonium sulfate + 50 μg lucanthone/ml.

in mammalian cells$^{15}$. Since this pathway seems crucial for the activation of lucanthone in the treatment of schistosomiasis$^{16}$ it is interesting that hycanthone has no effect on termination in vitro.

DISCUSSION

We have demonstrated that the ribosomal RNA synthesis preferentially is inhibited in isolated nuclei from Tetrahymena cells treated with low concentration of lucanthone (<5 μg/ml). The drug exerts a specific effect on termination in isolated r-chromatin causing the RNA polymerase to transcribe the spacer distal to the terminator. The effect of lucanthone on the termination process seems specific as we do not observe any effect on the elongation process.

It is difficult to predict a mechanism for the interaction
of lucanthone on the process of termination, as very little is known about this process in eukaryotic cells. Two mechanisms for termination of transcription have been described in prokaryotes: (i) a rho-independent and (ii) a rho-dependent termination as reviewed in ref. 17. Rho-independent termination is the result of an interaction between the RNA polymerase and the termination sequence on the template and/or specific structures of the 3'-end of the nascent RNA. In the rho-dependent termination, the translational machinery attached to the nascent RNA chains plays an important role in preventing intracistronic termination. Recently we have been able to isolate a protein factor responsible for termination of transcription on the r-chromatin (Leer, Tiryaki, and Westergaard, manuscript in preparation). This factor can be extracted from untreated nucleoli and it is present in more than one copy per gene. However, the presence of such a protein factor does not indicate that termination of transcription in this case parallels rho-dependent termination in prokaryotes as the transcription product, rRNA, is not translated. It is at the present unknown if the factor interacts with the DNA, with the nascent RNA chains or with the RNA-polymerase molecules. Further experiments with lucanthone and proflavine might give more detailed information about the termination process as it is plausible that the drugs suppress the normal termination by changing specific structures on the DNA template or on the RNA chains in such a way that the recognition sites for the terminator protein are distorted.

The present investigation shows that lucanthone in vitro interacts with the mechanism of termination allowing the endogenous RNA polymerases to read through the normal termination signal. Hybridization studies suggest that the drug exerts a similar effect in vivo. Therefore the preferential inhibition of the synthesis of ribosomal RNA in vivo might be due to the lack of reinitiating RNA-polymerase molecules caused by incomplete termination on the gene.

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