EFFECT OF PROTEIN SYNTHESIS INHIBITORS AND LOW CONCENTRATIONS OF ACTINOMYCIN D ON RIBOSOMAL RNA SYNTHESIS

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Received 20 August 1979

1. Introduction

It is known that low concentrations (0.001–0.05 μg/ml) of actinomycin D selectively inhibit ribosomal RNA (rRNA) synthesis when administered 'in vivo' [1,2]. This effect has been explained on the basis of a direct action of the drug on nucleolar transcription [3]. However, the same doses of actinomycin D that are effective in vivo do not inhibit RNA polymerase I (EC 2.7.7.6) activity when added to isolated nuclei although they do slightly decrease RNA polymerase II activity; this result suggested that the specific action of the antibiotic on rRNA synthesis might be indirect [4]. The observations that after in vivo administration of low doses of actinomycin D there is a decrease in the RNA polymerase II with a concomitant inhibition in the RNA polymerase I activity in the isolated nuclei and furthermore, that the time course of this inhibition is similar to that found after suppression of protein synthesis, lend support to the proposal that low doses of actinomycin D affect rRNA synthesis through inhibition of the synthesis of messenger RNAs (mRNAs) of high turnover which code for proteins required for nucleolar activity [5]. In this proposal the idea is implied that rRNA synthesis is under the control of mRNA synthesis.

In the forementioned studies rRNA synthesis was measured in isolated nuclei or nucleoli [4,5]. In these in vitro systems the problem of determining the specific activity of the nucleotide precursor pools [6,7] is overcome, and therefore they are widely used for studying transcription of ribosomal genes [4,5,8–13]. However, the rate of elongation by RNA polymerase I is only 1–2% that in vivo [9]. On the other hand, one has to take into consideration the side effects that administration of different antibiotics might exert in the in vitro assays. With these considerations in mind we decided to study the effect of protein synthesis inhibitors and low doses of actinomycin D on rRNA synthesis by measuring transcription both in vivo and in vitro.

2. Materials and methods

2.1. *Obtaining Ehrlich ascites cells*

The cells used were grown for 5–7 days in the abdominal cavity of mice. The preparation of the cells was carried out as [11]. Cells were incubated at 2×10^6/ml in Eagle's medium.

2.2. *Nuclear isolation*

Nuclei were purified as in [12]. All solutions contained 1 mM phenylmethanesulfonylfluoride (Sigma).

2.3. *DNA-dependent RNA polymerase I assay*

RNA polymerase I in isolated nuclei was assayed by measurement of the incorporation of [^{3}H]UTP (New England Nuclear) into RNA as in [12]; the assay solution contained phenylmethanesulfonylfluoride (0.05 mM); α-amanitin, (200 μg/ml) was added to suppress RNA polymerase II and III activities [14]. When heparin (700 μg/ml) was included in the assay α-amanitin was reduced to 5 μg/ml since no RNA polymerase III activity was detected when heparin is present (results not shown). Except for the experiments in fig.1 the incubations were done for 8 min at 37°C.

2.4. *Labeling and analysis of the RNA*

Cells were labeled with the [^{3}H]nucleoside (New England Nuclear) for 10 min; incorporation was halted and the RNA was purified as in [12]. The pelleted RNA was dissolved in a buffer containing: 10 mM Tris–HCl (pH 7.6); 1 mM ethylenediamine-tetraacetic acid; 100 mM NaCl; 0.5% sodium dodecylsulfate. Amounts of RNA corresponding to ~1 × 10^6 cells were layered on a 15–30% sucrose gradient in the same buffer and centrifuged at 39 000 rev./min for 4 h and 30 min at 25°C in a MSE-SW 40 rotor. The gradients were fractionated and the trichloroacetic acid-precipitable radioactivity in each fraction was determined [15].

3. Results and discussion

3.1. *Effect of addition of different antibiotics to whole cells on the in vitro assay of nuclear RNA polymerase I*

The effect of a low concentration of actinomycin D or of pactamycin administered in vivo on the RNA polymerase I activity in isolated nuclei is shown in fig.1. The kinetics of UMP incorporation is similar in nuclei from control and pactamycin-treated cells. The reaction stops very soon in both cases, thus suggesting that the elongation of RNA chains is the main activity that isolated nuclei retain. The initial rate of transcription and final plateau level in nuclei from cells in which protein synthesis have been inhibited, is ~50% as compared to control nuclei. These results are in agreement with [11,13]. Nuclei from cells incubated with the concentration of actinomycin D reported to selectively suppress rRNA synthesis [1,2], showed a substantial enzyme I activity and, unlike the usually observed kinetics, the incorporation of [^{3}H]UMP was linear (fig.1A). To determine whether this is due to reinitiation of RNA chains, polymerase I activity was assayed in the presence of heparin. This drug inhibits free RNA polymerase molecules but has no effect on those already engaged in transcription [9,16]. The results in fig.1B show that heparin abolishes polymerase I activity in nuclei from cells treated with actinomycin D. On the contrary, in nuclei isolated from control and pactamycin-treated cells, UMP incorporation is enhanced by the drug. At the concentration used in this study, heparin disrupts nuclear structure, and the enhancement in transcription is probably similar to that produced by high ammonium sulfate [16]. It is worth noting that the decrease in the transcriptional capacity of nuclei after protein synthesis inhibition is almost the same whether polymerase I is assayed with or without heparin.

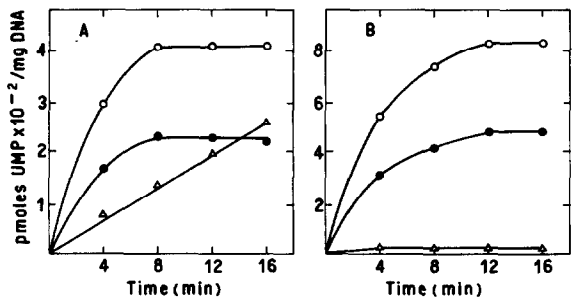

Fig.1. Kinetics of RNA polymerase I reaction in nuclei from cells treated with different antibiotics. Cells were incubated for 2 h and 30 min (–○–), or for 2 h, at which time actinomycin D (0.05 μg/ml) (–△–) or pactamycin (0.3 μg/ml) (–●–) was added and incubation continued for 30 min. After incubation nuclei were isolated and RNA polymerase I activity was assayed without (A) or with (B) heparin.

3.2. *Effect of different antibiotics on rRNA synthesis in whole cells*

The effect of a low concentration of actinomycin D or pactamycin on rRNA synthesis measured in whole cells is shown in fig.2. After treatment of the cultures for 20 min with the inhibitors, the cells were labeled with a pulse of [^{3}H]guanosine and the purified RNA was analyzed on sucrose gradients. The RNA patterns showed that after inhibition of protein synthesis there is a substantial incorporation of label into the 45 S RNA peak which amounts to ~50% of that in control cells. This result is in good agreement with that of in vitro transcription. In the actinomycin D-treated cells, no peak of radioactivity could be detected in the 45 S region, a finding which correlates with the absence of polymerase I activity in the isolated nuclei when heparin is included in the assay (fig.1B). The correlation between in vivo and in vitro results when initiation of RNA chains is suppressed by heparin was confirmed in the experiment depicted in fig.3, in which the concentration range of actinomycin D that inhibits rRNA synthesis was studied. These observations suggested that the polymerase I

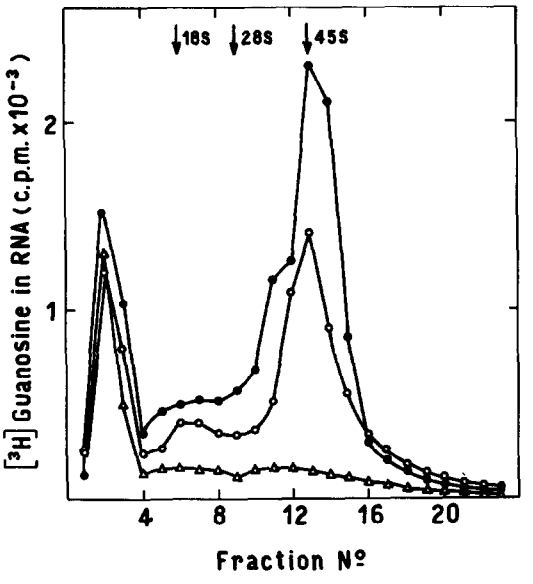

Fig.2. Sedimentation velocity analyses of labeled RNAs from cells treated with different antibiotics. Cells were incubated for 2 h and at this time actinomycin D (0.05 μg/ml) (–△–), or pactamycin (0.3 μg/ml) (–○–) was added; after further incubating for 20 min cells were pulse-labeled with [^{3}H]guanosine (60 μCi/ml). Control cells were incubated for 2 h and 20 min and pulse-labeled as before (–●–); RNAs were purified and analyzed as in section 2.

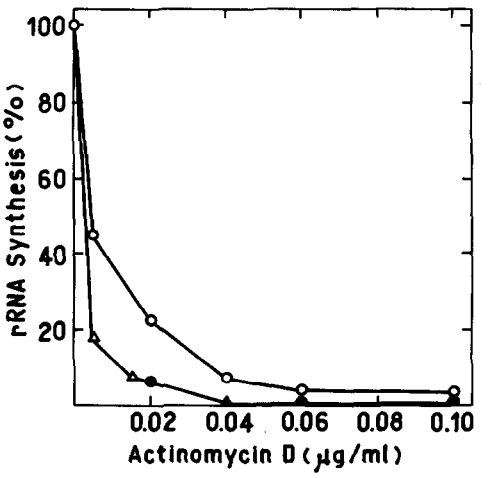

Fig.3. Effect of addition of different concentrations of actinomycin D to whole cells on rRNA synthesis. Cells were incubated for 2 h and then exposed to different concentrations of actinomycin D for 30 min. At this time, cells were harvested from an aliquot of the culture, nuclei were isolated and RNA polymerase I activity was assayed in the presence of heparin (–○–). To the remaining incubate, [^{3}H]uridine (30 μCi/ml) was added and incubation was continued for 10 min; at this time the RNA was purified and analyzed as in section 2; the radioactivity in the 45 S rRNA peak was determined graphically [12] (–●–). For comparison, the data of [1] on the inhibition of rRNA synthesis by actinomycin D in HeLa cells are included (–△–).

activity observed in nuclei from cells treated with a low amount of actinomycin D (fig.1A) most probably arises from non-specific initiations of RNA chains. A possible explanation for this result is based on the fact that administration of low doses of actinomycin D induce distortion of the helix and extensive DNA breakage [17], thus creating pseudo-initiator sites for molecules of polymerase I not already engaged in transcription.

3.3. *Time course of the inhibition of rRNA synthesis by the different drugs*

Once the conditions for measuring rRNA transcription in vitro were established, the time course of the inhibition of rRNA synthesis by the different drugs was studied. If low concentrations of actinomycin D affect rRNA synthesis through inhibition of the synthesis of mRNAs which code for proteins required for nucleolar activity one might expect that the effect of protein synthesis inhibitors should be manifested earlier than that of a low dose of actinomycin D. As shown in fig.4A, a low concentration of actinomycin D causes a very rapid decrease in rRNA synthesis, while the decay observed after inhibition of protein synthesis with either pactamycin or cycloheximide is manifested later. That this is not the consequence of a delay in the inhibition of protein synthesis is illustrated in fig.4B, where a rapid shut off of labeled leucine incorporation after administration of the antibiotics is seen.

These results do not support the view that low doses of actinomycin D affect rRNA transcription through inhibition of the synthesis of certain mRNAs. The very early effect of actinomycin D suggests a direct action of the drug although the data do not preclude the possibility of a specific inhibition of nucleoplasmic transcripts directly affecting nucleolar function. In this regard, it has to be pointed out that up to now there is no conclusive evidence for an

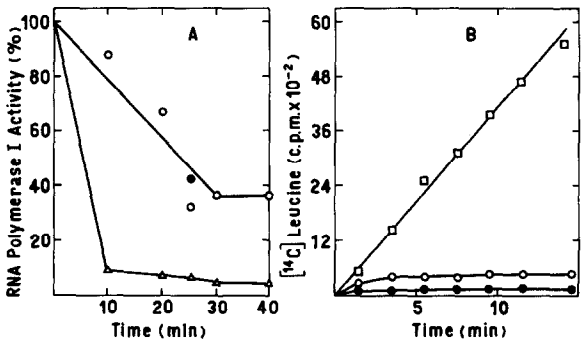

Fig.4. Time course of the effect of actinomycin D and protein synthesis inhibitors on rRNA synthesis. (A) Cells were preincubated for 2 h, at which time (time zero), pactamycin (0.3 μg/ml) (–○–), cycloheximide (30 μg/ml) (–●–), or actinomycin D (0.04 μg/ml) (–△–) was added and incubation was continued. At the times indicated nuclei were isolated and polymerase I was assayed in the presence of heparin. (B) Cells were incubated for 2 h at which time pactamycin (0.3 μg/ml) (–○–) or cycloheximide (30 μg/ml) (–●–) was added and 30 s later 1.2 μCi/ml of [^{14}C]leucine. Control cells were incubated for 2 h and labeled as before (–□–). After incubating for the times indicated the radioactivity incorporated into proteins was measured as in [11].

extranucleolar control of rRNA synthesis, since the experiments in which α-amanitin was administered in vivo to specifically suppress nucleoplasmic transcription gave conflicting results [18].

Acknowledgements

This work was supported in part by grants from Secretaría de Ciencia y Técnica, Fundación Alberto J. Roemmers and Liga Argentina de Lucha contra el Cáncer. We wish to thank Professor Th. Wieland for providing us the α-amanitin and Drs I. Algranati and A. C. Paladini for the critical revision of the manuscript. M. T. F. F. is career investigator of CONICET.

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