CONTROL OF rRNA SYNTHESIS: EFFECT OF PROTEIN SYNTHESIS INHIBITION

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1. Introduction

Synthesis of 45S ribosomal RNA (rRNA) precursor falls steeply after protein synthesis inhibition in HeLa and rat liver cells [1,2]. Moreover, there is a rapid decline in the activity of the enzyme that transcribes rRNA (RNA polymerase 1) in Ehrlich ascites and rat liver nuclei after the administration of cycloheximide or pactamycin [3–5]. From these results it has been postulated that a short-lived protein(s) is required for the transcription of the nucleolar genes [1–5]. This protein(s) is not the RNA polymerase 1 itself, as is shown by the fact that the same amount of enzyme is isolated from both control and protein synthesis inhibited Ehrlich ascites and rat liver nuclei [5,6]. The regulation might be mediated, instead, by a highly turning over protein(s) that would control the RNA polymerase 1 capacity to transcribe ribosomal genes [6].

We may now ask whether this control is effected by varying the number of RNA polymerase molecules in activity or by regulating the rate of transcription of the genes coding rRNA. With a view to deciding between these possibilities we have compared the time required to synthesize the rRNA precursor in control and protein synthesis-inhibited Ehrlich ascites cells.

2. Materials and methods

The isolation of Ehrlich ascites tumour cells and the preparation of the incubation medium (Eagle's medium) were carried out as described previously [7]. Cells were incubated at a concentration of 1×10^6/ml medium.

2.1. *Radioactivity in the rRNA precursor*

Ehrlich ascites cells were incubated with 40 μCi/ml of [^{3}H]uridine. At the times indicated the incorporation was stopped by pouring 1 ml of the incubate onto 10 ml of crushed, frozen Dulbecco's medium [8]. The cells were collected by centrifugation and dissolved in 2 ml of a buffer containing: 0.1 M NaCl; 0.1 M Tris–HCl pH 7.20; 0.001 M EDTA and 0.5% sodium dodecyl sulfate. At this stage, a tracer amount of ^{14}C-labelled rRNA was added to correct for small differences in recovery during RNA isolation and analysis. The polynucleotides were extracted with a mixture of phenol and chloroform as described by Perry et al. [9]. To the combined aqueous phases, 2.5 volumes of ethanol were added and the mixture was allowed to stand overnight at –20°C. The precipitate was collected by 30 min centrifugation at 12 000 *g*. The pelleted polynucleotides were dissolved in 1 ml of ten times concentrated buffer RSB [10] and the DNA was digested by 4 min incubation at 37°C with about 100 μg of deoxyribonuclease (Worthington, electrophoretically purified). A 0.1 M solution of EDTA was then added (0.15 ml) followed by a 10% sodium dodecylsulfate solution so as to attain a concentration of 0.5%. The RNA was extracted by shaking with an equal volume of phenol at room temperature. To the aqueous phase, 2.5 vol of ethanol were added and the RNA was pelleted as described above. The pellet was dissolved in 0.2 ml of the electrophoresis buffer containing 30% sucrose. The RNA from about $2.5–3.5 \times 10^5$ cells was analyzed by electrophoresis in 2.5% acrylamide gels in accordance with Loening [11]. The electrophoreses were performed at 6 mA per gel for 5 hr. The gels were sliced and counted as

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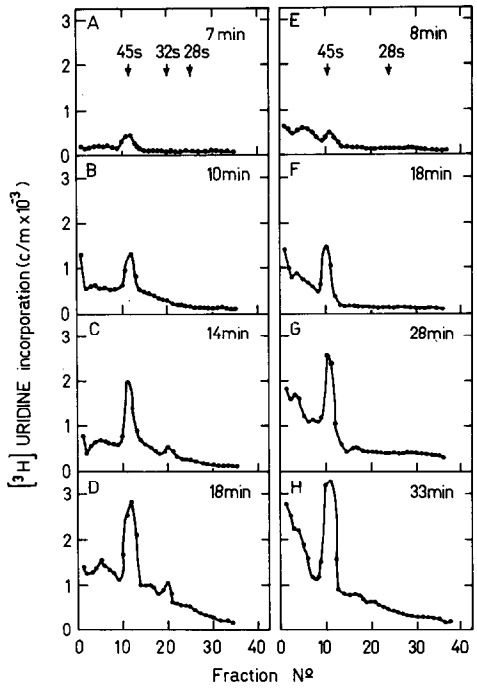

Fig.1. A typical analysis of rRNA by polyacrylamide gel electrophoresis. A to D: Ehrlich ascites cells were incubated in Eagle's medium for 90 min. [^{3}H]uridine was then added and incubation was continued for the times indicated. E to H: After incubation of the cells for 90 min in Eagle's medium, 0.25 μg/ml of pactamycin was added and the incubation was continued for 15 min. At this point, the cells were labelled with [^{3}H]uridine for the times indicated. The inhibition of protein synthesis by that concentration of pactamycin was more than 98%. The RNA was extracted and analyzed as indicated in Methods.

described by Weinberg [12]. The radioactivity in the rRNA precursor peaks was estimated graphically [13]. The radioactivity in the 32S rRNA precursor (see fig.1C and D) was expressed as 45S RNA by multiplying the counts in the 32S peak by a factor of 1.87. This factor is the ratio between the mol. wts of the 45S and 32S molecules [14].

2.2. *Radioactivity in total RNA*

Uridine incorporation into RNA was determined by adding 3 μCi of [^{3}H]uridine per ml of medium. At the times indicated aliquots of 0.2 ml were poured onto crushed, frozen Dulbecco's medium and the cells were deposited by centrifugation. A small volume of water was added to lyse the cells followed by 5–7 ml of cold 5% trichloroacetic acid. The precipitate was collected by centrifugation and washed three times with cold 5% trichloroacetic acid. The pellet was dissolved in 0.2 ml N.C.S. (Amersham/Searle) and counted in toluene scintillation fluid. The assays were performed in duplicate.

3. Results and discussion

A study of the kinetics of incorporation of a radioactive precursor into fully transcribed RNA molecules affords an estimate of the time required to synthesize the RNA chain. Radioactivity in whole molecules accumulates as the square of the labelling time up to one transcription time and is linear thereafter. The extrapolated linear portion intersects the time axis at one half the transcription time [10,15,16].

In this work we have measured the incorporation of a radioactive nucleotide into the 45S rRNA precursor in Ehrlich ascites cells, incubated with and without the protein synthesis inhibitor pactamycin. From the data obtained we have estimated the time required to transcribe the rRNA precursor in both control and pactamycin-treated cells.

Fig.1 illustrates a typical analysis of the labelled RNA by polyacrylamide gel electrophoresis. At 14 and 18 min pulses of [^{3}H]uridine, a small peak of radioactivity appeared at the 32S position in the gels from control cells. No 32S peak appeared, even after 30 min of label, in the gels from cells incubated with pactamycin. This result is in agreement with observations that in HeLa and L cells there is an aberration in the processing mechanism of the 45S rRNA after protein synthesis inhibition with cycloheximide [1,17].

The graph of [^{3}H]uridine incorporated into the rRNA precursor as a function of the labelling time in cells incubated without pactamycin is illustrated in fig.2. The radioactivity increases linearly after an initial delay of about 5.5 min. This lag reflects the time at which linear incorporation of the label into total RNA begins plus the time required to transcribe a complete 45S RNA chain. As shown in fig.3, incorporation of [^{3}H]uridine into total RNA is linear after a lag of 2 min. The transcription time

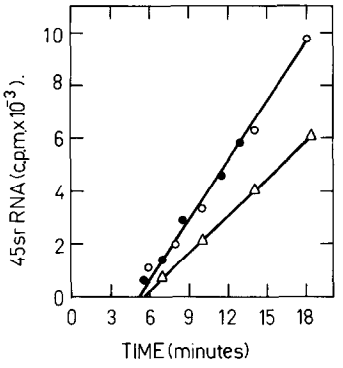

Fig.2. Incorporation of [^{3}H]uridine into the rRNA precursor in Ehrlich ascites cells. The cells were labelled after 90 min of incubation in Eagle's medium. The amount of radioactivity in the rRNA precursor peaks was estimated as described in Methods. The different symbols correspond to independent experiments.

of the rRNA precursor is then twice the difference between the time lag found in the 45S species alone (fig.2) and that in total RNA (fig.3), or about 7 min [10]. This value is in close agreement with the one of 5 min estimated for the rRNA precursor synthesis in growing fibroblasts in culture [18].

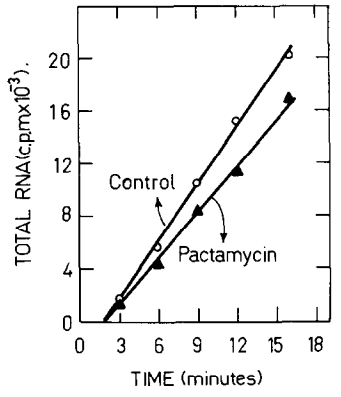

Fig.3. Incorporation of [^{3}H]uridine into total RNA. Ehrlich ascites cells were incubated for 90 min in Eagle's medium. At this moment the incubate was divided into two equal parts. Pactamycin (0.25 μg/ml) was added to one and both were incubated for another 15 min. The cells were then labelled for the times indicated. Samples were taken, poured on cold Dulbecco's medium and processed as described in Methods. Cells incubated without (○) and with (▲) pactamycin. Incorporation of [^{3}H]uridine in both control and pactamycin treated-cells is linear for at least 30 min (results not shown).

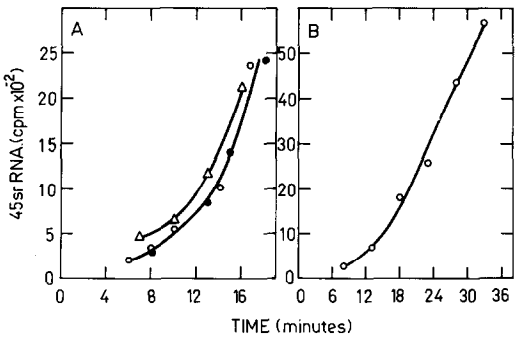

Fig.4. Incorporation of [^{3}H]uridine into the rRNA precursor in protein synthesis-inhibited Ehrlich ascites cells. After incubating the cells for 90 min in Eagle's medium, pactamycin was added (0.25 μg/ml) and the incubation was continued for 15 min. The cells were then labelled. The amount of radioactivity in the rRNA precursor peaks was estimated as stated in Methods. The different symbols correspond to independent experiments.

In contrast with the results obtained in control cells, [^{3}H]uridine incorporation into 45S rRNA is not linear for labelling times under 18 min in cells incubated with pactamycin (fig.4,A). The apparently increasing rates of rRNA precursor synthesis are consistent with the idea that the labelling times are short in relation to the time required to transcribe the 45S RNA chain [15,16]. When the pactamycin-treated cells are labelled for longer periods, incorporation becomes linear and extrapolates back to a time of about 12 min (fig.4,B). Since the time at which linear incorporation of [^{3}H]uridine into total RNA begins is identical in both control and pactamycin treated cells (2 min; fig.3), it was estimated that the time required to synthesize the complete 45S rRNA chain after a short period of protein synthesis inhibition is about 20 min or three times as long as in control cells. These results strongly suggest that a short-lived protein(s) controls the rate of transcription of the genes coding rRNA.

Just how transcription is changed is an open question. A possibility is that the rate of polymerization of nucleotides by RNA polymerase I may be controlled. In this regard it is worth noting that after protein synthesis inhibition the RNA polymerase 1 activity in Ehrlich ascites nuclei declines to about 1/3 of its original value [3]. Since RNA chain elongation

is the main activity that the isolated nuclei display, this and the above results taken together suggest that the rate of polymerization of nucleotides might be controlled.

It has been reported that the time required to synthesize the rRNA precursor is 3 to 4 times longer in resting than in growing fibroblasts [18]. On the other hand, in uterine and adrenal nuclei from both control and hormone-treated rats, the number of growing RNA chains is identical, although the incorporation of a radioactive nucleoside triphosphate into the internucleotide position of the chains is enhanced by the hormone. In these nuclei systems, mainly rRNA synthesis was measured [19,20]. All these results suggest that regulation of rRNA synthesis at the level of chain elongation is a general phenomenon. A short-lived protein might be involved in the control of this process.

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