Volume 12 Number 7 1984 Nucleic Acids Research

Interrelations between the maturation of a 100 kDa nucleolar protein and pre rRNA synthesis in CHO cells

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Received 13 February 1984; Accepted 8 March 1984

ABSTRACT

The synthesis of preribosomal RNA is inhibited "in vivo" and "in vitro" by the protease inhibitor leupeptin. "In vivo" leupeptin decreases by 74 % the incorporation of labeled uridine into 45S pre rRNA while the synthesis of other RNA species is only slightly decreased. "In vitro", the elongation of already initiated pre rRNA chains that is achieved by incubation of isolated nucleoli is blocked by leupeptin. On the other hand, "in vitro" leupeptin has no direct effect on RNA polymerase I, tested in a nonspecific transcriptional system with Calf thymus DNA as template and in run off experiments with a cloned DNA containing the initiation site of the rDNA gene. A 100 kDa nucleolar protein which has been shown to be endoproteolytic cleaved "in vivo" (1) acts as an inhibitor of rDNA transcription in presence of leupeptin but produces little effect on the nonspecific transcription. In absence of the drug, the 100 kDa protein is processed in specific peptides which appeared to be similar to the "in vivo" maturation products. The possible role of the 100 kDa maturation process in the regulation of rDNA transcription is discussed.

INTRODUCTION

Several factors have been proposed to play a role in the regulation of pre rRNA synthesis (2). In "in vivo" experiments, correlations have been established between pre rRNA synthesis and the evolution of given parameters such as : aminoacid pool size (3, 4), nucleotide pool size (5), turn over of labile stimulatory protein (6) in particular ornithine decarboxylase (7), and phosphorylation of specific proteins (6). The recent development of "in vitro" systems in which a defined rDNA fragment containing the "in vivo" start site can be transcribed by RNA polymerase I, should allow the characterization of factors involved in the regulation of pre rRNA synthesis (8, 9).

Such regulatory proteins must be localized in the nucleolus, a large subnuclear structure which contains most of the actively transcribed ribosomal genes in exponentially growing eukaryotic cells (10). The nascent transcripts are immediately associated with ribosomal proteins (11) and

with a class of proteins that turn over in the nucleolus and are not recovered in cytoplasmic ribosomes (12). Among these proteins, a 100 kDa species, particularly abundant in exponentially growing cells, is in amount that can be directly related to the level of 45S pre rRNA synthesis (13). Recently protein C23, that behave like the 100 kDa protein, was shown to bind to DNA and in particular with a high affinity to sequences of the nontranscribed spacer (14). An over property of the 100 kDa protein is its ability to be cleaved into specific maturation products during preribosome biogenesis. Correlations have been established between this maturation process and the transcription of pre rRNA (1, 15). Taken together these results suggest that the 100 kDa protein itself and/or the process of its maturation may play a role in the regulation of ribosomal gene transcription.

In this report, we have studied the interrelations between the process of maturation of the 100 kDa protein and the synthesis of pre rRNA. We have used the protease inhibitor leupeptin (16) which penetrates into the cell and blocks the protease activity associated with the 100 kDa protein both "in vivo" and "in vitro" (15). "In vivo" leupeptin induces the accumulation of the 100 kDa, and inhibits the synthesis of the 45S pre rRNA. "In vitro", the protein acts as an inhibitor of transcription in presence of leupeptin.

MATERIALS AND METHODS

Cell culture and fractionation.

Chinese hamster ovary cells were grown in monolayer culture in Falkon flasks (17). Prior to labeling, cells were incubated for 1 h in Eagle's minimum essential medium depleted in phosphate (1/100 of control). Cells were labeled for 10 min to 1 h in the same medium containing ^{32}P orthophosphate (40 µCi/ml; 50 Ci/mg, Amersham, England). Labeled or unlabeled cells were harvested and fractionated into nuclei and nucleoli according to Zalta et al. (18). RNAs were extracted as previously described (17) and were fractionated by electrophoresis on 0.8 % agarose gels containing 10 mM methyl mercuric hydroxide in borate buffer (19). After electrophoresis the gels were dried and autoradiographed.

"In vitro" synthesis of RNA.

Nucleoli were incubated in a medium containing 100 mM Tris HCl pH 8.5, 6 mM NaF, 5 mM Mg acetate, 50 mM $(NH_4)_2SO_4$, 10 mM dithiothreitol, 10 % glycerol, 300 µM ATP, GTP, UTP, 7 µM CTP and 5 µCi ^{32}P CTP (400 Ci mM^{-1}).

After incubation RNAs were extracted by precipitation in lithium-urea (20). RNA polymerases were extracted from purified nuclei by high salt (21). RNA polymerase I activity was determined in presence of 300 µg/ml ∝-amanitin.

The mouse rDNA recombinant plasmid pMr SP carrying the transcription initiation site was a gift from I. Grummt (22). Extracts containing the RNA polymerase I and the initiation factors, were prepared from 10^9 nuclei or nucleoli isolated according to Zalta et al. (18) in absence of Ca^{2+}. The protein solubilization was carried out as described by Dignam et al. (23) in 3 ml of 20 mM Hepes (pH 7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 25 % glycerol (v/v). After centrifugation 30 min. at 25000g, the supernatant was dialyzed against 50 vol of 20 mM Hepes (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT and recentrifuged for 20 min. at 25000 g. The supernatants were called nuclear and nucleolar extracts and contained respectively 8 mg and 2 mg protein per ml. "In vitro" RNA synthesis was carried out at 30°C for 50 min. in a 5 µl reaction mixture as described by Mishima et al (9), in presence of 20 µl and 10 µl of nuclear and nucleolar extracts respectively. After addition of 0.3 % sodium dodecyl sarcosinate, 0.15 M NaCl and 5 µg yeast tRNA, ^{32}P RNAs were extracted twice with phenol-chloroform, precipitated with ethanol and analysed by gel electrophoresis.

Protein analysis

The nuclear proteins were separated by electrophoresis on 10-16 % polyacrylamide gradient slab gels. Part of the gel was stained with Coomassie blue, while proteins of the other part were electrotransferred to nitrocellulose filters (1). The filters were then incubated with an antiserum raised against the 100 kDa nucleolar protein and the immunocomplexes were detected with ^{125}I labeled protein A (1, 24). The filters were washed, dried and autoradiographed.

RESULTS

Effects of leupeptin on pre rRNA synthesis "in vivo"

Exponentially growing CHO cells were incubated for 1 h with ^{32}P orthophosphate in the presence or the absence of leupeptin. RNAs were extracted from purified nucleoli and analysed by electrophoresis under denaturing conditions. As shown in fig. 1, in both treated and untreated cells, 45S, 32S pre rRNAs and 28S rRNAs were labeled though leupeptin decreases dramatically the incorporation of ^{32}P orthophosphate.

To determine accurately the amount of label in each RNA species, bands were cut out of the agarose gels and incorporated radioactivity was counted

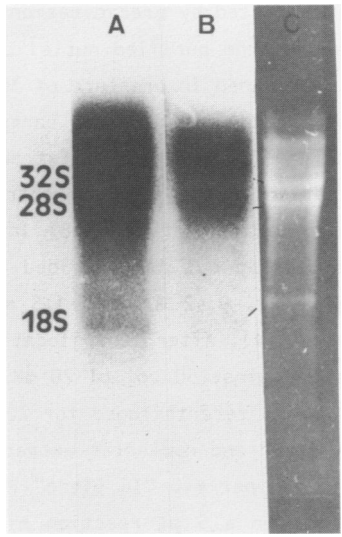

Figure 1 : "In vivo" effects of leupeptin on pre rRNA synthesis.
Exponentially growing CHO cells were incubated for 1 h in the presence of ^{32}P orthophosphate with or without 10 µg/ml leupeptin. Nucleoli were prepared and RNA extracted as described in materials and methods. RNAs were analysed by electrophoresis on 0.8 % agarose gels containing 10 mM methyl mercuric hydroxide (19). After electrophoresis the gel was stained with ethidium bromide to detect the 28S and 32S RNAs and autoradiographed (A, B).
A : control cell - B : 10 µg/ml leupeptin - C : EtBr stained gel.

(table 1). In the presence of leupeptin (10 µg/ml) the relative inhibition of incorporation is higher for the 45S RNA than for the 28S RNA : (76 % and 53 % respectively). At 50 µg/ml of leupeptin a decrease in the incorporation of ^{32}P orthophosphate into the 45S RNA is still observed, but more label is recovered in the 28S and 18S RNA. This paradoxical effect of leupeptin in vivo according to concentration is also detectable at 10 µg/ml in function of treatment time. After 1 h incubation, the pre rRNA synthesis gradually recovered and the addition of fresh leupeptin to the medium has no further effect.

"In vitro" effects of leupeptin on the synthesis of RNA

Isolated nucleoli were incubated "in vitro" in the presence of ^{32}P labeled CTP in a medium that allowed the elongation of preinitiated pre rRNA molecules and the accurate termination of transcription. Fig. 2 shows that in the presence of RNasine, a strong RNase inhibitor most of the label is recovered in the 45S pre rRNA. Addition of leupeptin decreases the in-

TABLE 1 : Effects of leupeptin on the synthesis of pre rRNAs.

RNA species	Control	Leupeptin (10 ug/ml)	Leupeptin (50 ug/ml)
45S	4 220	1 000 (23.6)	3 520 (83.4)
36S	4 820	830 (17.2)	3 060 (63.5)
32S	2 650	740 (27.9)	3 360 (126.8)
28S	1 500	700 (46.6)	2 350 (156.7)
18S	770	370 (48.)	940 (122.)
$\sum$cpm	13 960	3 640 (26.)	13 230 (94.)

Cells were labeled for 1 h with ^{32}P orthophosphate. Nucleoli were then prepared and RNAs extracted as described in Materials and Methods. RNAs were fractionated as in fig. 1 and bands of the gel were cut out. After hydrolysis, the radioactivity in each RNA species was determined. Results are expressed as counts incorporated in equivalent amounts of total nucleolar RNA determined by scanning of the ethidium bromide stained gel. The values in brackets are the percent of incorporated counts in control cells.

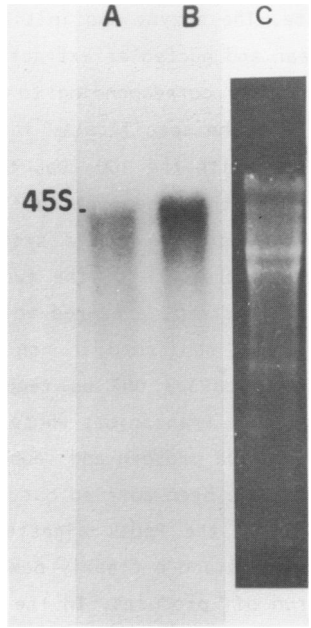

Figure 2 : Effects of leupeptin on RNA synthesized "in vitro" in isolated nucleoli.

2.10^7 nucleoli (100 µg RNA) were isolated and separated in 2 fractions. Each was incubated for 15 min. in the transcription medium with ^{32}P CTP in presence (A) or absence (B) of 10 µg/ml leupeptin. RNAs were then extracted and analysed as described in the legend of fig. 1.
C : EtBr stained gel.

corporation of labeled CTP by 50 %. In the absence of RNasine, part of the label comigrates with the background of randomly sized molecules but the addition of leupeptin results in the same decrease of CTP incorporation.

To test whether leupeptin acts directly on RNA polymerase I, the enzyme was extracted from isolated nuclei at high salt, and tested in presence of 300 µg/ml ∝ -amanitin with calf thymus DNA as template. The addition of leupeptin has no effect on the amount of RNA synthesized in this system.

Effects of a 100 kDa nucleolar protein and leupeptin on specific "in vitro" transcription of rDNA.

In previous experiment, we have shown that a major nucleolar protein (100 kDa), is cleaved "in vivo" in several peptides which are associated with preribosomes (1). This maturation process is inhibited by leupeptin. Since this protein also bind to DNA, we have checked its potential effects in run off experiments with a cloned DNA (SalI-PvuII) containing the 5'end of ribosomal gene as template. The enzyme and initiation factors were contained in a mixture of nuclear and nucleolar extracts. As shown in fig. 3A, two main species were synthesized corresponding to the full length transcript (SalI-PvuII : 454 nu) and the specifically initiated transcript (296 nu). To perform the experiments with the 100 kDa, the DNA was first incubated with the nuclear extracts, then the protein (1 ug) was added with or without leupeptin. A dramatic decrease in the synthesis of the specific transcript was only observed in presence of the two components, while the synthesis of the full length transcript appeared to be less affected (fig. 3B, C). This finding was further confirmed in non specific transcription assays using a HinfI digest of pBR322 DNA as template. Most of the DNA fragments gave rise to full length transcripts whose synthesis was insensitive to the presence of the 100 kDa protein and leupeptin (fig. 4A).

Two additional controls have been carried out to further characterize our transcriptional system using the PvuII digested pMr DNA as template (fig. 4B). A comparison of lanes a and b clearly demonstrate that leupeptin alone has no effect on the run off products. In the other experiment, were compared the relative efficiencies of the 100 kDa protein and histones that bind unspecifically to DNA, to inhibit the specific rRNA synthesis. To get a similar level of inhibition, the ratio protein/DNA must be 10 times higher with the histones than with the 100 kDa and leupeptin (fig. 4B, C-E).

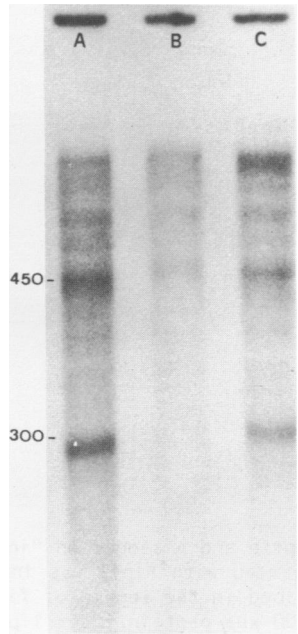

Figure 3 : Effect of the 100 kDa nucleolar protein and leupeptin in run off experiments.

1 µg of the pMr SP DNA plasmid digested with SalI and PvuII restriction enzymes was used in each assay (22). DNA was incubated for 30 min. at 30°C in the complete transcription medium without the labeled CTP. Then 1 µg of the 100 kDa protein and ^{32}P CTP were added and incubation was extended for 50 min. After synthesis, the RNA was purified and analysed on a 5 % acrylamide urea gel. The 100 kDa protein has been purified from nucleoli as previously described (15).
A : control - B, C : in presence of the 100 kDa - B : 100 kDa + 10 µg/ml leupeptin. Nucleotide number of the transcripts specifically initiated (296) - Full length SalI-PvuII fragment (454).

"In vitro" maturation of the 100 kDa protein during transcription

In order to follow the eventual maturation of the 100 kDa protein during the "in vitro" transcription of rDNA, proteins from the run off assay were analyzed before, during and after incubation. To detect the maturation products from among the other proteins contained in the nuclear extract an antiserum raised against the 100 kDa protein was used (1). Proteins from the slab gel were electrotransferred onto nitrocellulose paper. After immunoreaction bound antibodies were detected with iodinated protein A. As shown in fig. 5, the 100 kDa is cleaved into three major species of 95 kDa,

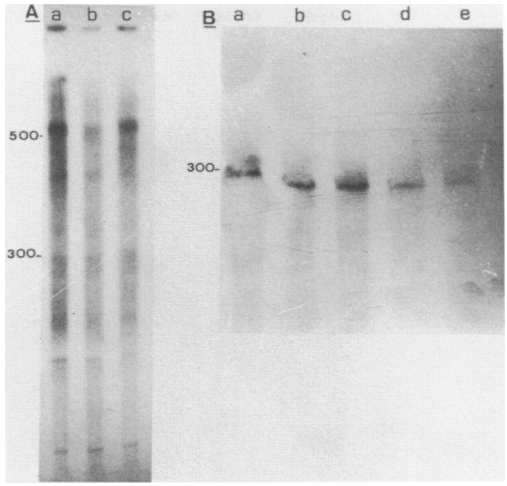

Figure 4 : Effects of leupeptin and histones on "in vitro" transcription.
A : 0.5 µg of DNA digested with HinfI was incubated with nuclear and nucleolar extracts as described in the legend of fig. 3.
a : control - b : 1 µg of 100 kDa protein - c : 1 µg of 100 kDa protein and 10 µg/ml leupeptin.
B : 0.5 µg of pMR SP plasmid linearized with PvuII was used as template.
a : 10 µg/ml leupeptin - b : control - c-e :in presence of histones (0.5 µg - 1 µg - 2 µg).

76 kDa and 70 kDa. Maturation of the 100 kDa protein continued throughout incubation but some undegraded protein was still present at the end of the assay. The addition of leupeptin almost completely blocks the maturation process (fig. 4C).

DISCUSSION

We have established a correlation between the level of pre rRNA synthesis and the maturation process of a 100 kDa nucleolar protein. The inhibition of the endoproteolytic cleavage of the 100 kDa protein "in vivo" or "in vitro" in isolated nucleoli is followed by a decrease of the rDNA transcription. The 100 kDa protein is multifunctional and its physicochemical characteristics suggest that it is similar to the nucleolar protein C_{23} of Novikoff hepatoma cells (26). The 100 kDa protein is present in most eukaryotic cells and has been highly conserved during evolution. An antibody raised against this protein extracted from CHO cells cross reacts with that of several other cell species. If its level in nucleoli appears clear-

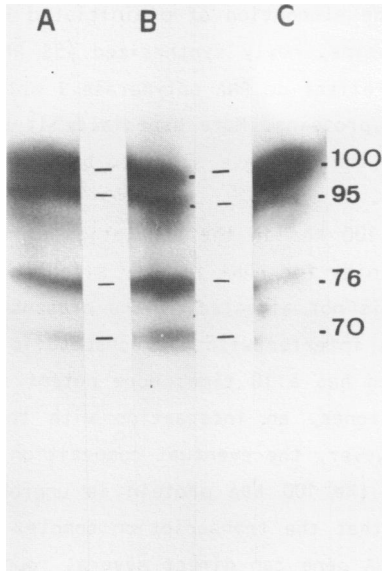

Figure 5 : Maturation of the 100 kDa protein during the run off experiments.

2.4 µg of 100 kDa protein was added to a run off experiment (fig. 3 : A, D). Proteins were then analysed on a polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (14). The filter was then incubated with an antiserum raised against the 100 kDa protein and the immunocomplexes were detected with ^{125}I labeled protein A. Incubation at 30°C. A : 25 min - B and C : 50 min - C : in presence of 10 µg/ml leupeptin.

ly to be related with the level or ribosomal RNA synthesis (13), little is known about the rate and the role of its endoproteolytic cleavage.

Leupeptin appears to be a valuable tool to study "in vivo" the role of protein maturation. The observed paradoxical effect in function of drug concentration must be brought together with the results of Sutherland et al. (27). They showed that according to concentration time of treatment and cell species, leupeptin acts "in vivo" as an inhibitor or an activator of catephsin B activity. Furthermore, Furuno et al. (28) have observed, in presence of leupeptin, the movement of lysosomal proteases into autolysosomes. Our results suggest that in CHO cells, leupeptin induces the cytoplasmic release of a lysosomal protease that further inactivates the drug.

The effects of leupeptin on rDNA transcription was clearly demonstrated in the "in vitro" experiments involving isolated nucleoli. In this

experimental system, the elongation of preinitiated RNA chains occurs and in the presence of RNasine, newly synthesized 45S RNA accumulates. Since the drug has no direct effect on RNA polymerase I activity, leupeptin must act on other nucleolar proteins. More especially it blocks the maturation of the 100 kDa protein that we have shown to be processed during preribosome biogenesis (1, 15).

The role of the 100 kDa in the regulation of rDNA transcription is still unclear. Its affinity for rDNA is only slightly higher than for random DNA sequences and is not affected by the presence of leupeptin. Since on one hand, it did not interfer with the nonspecific transcription and on the other hand it acted has a 10 times more potent inhibitor of specific transcription than histones, an interaction with the initiation factors must be considered. However, the eventual competition between the specific initiation factors and the 100 kDa protein is unprobable since a recent report (29) has shown that the transcription complex is highly stable and that each activated rRNA gene can direct several rounds of elongation and reinitiation.

The presented results demonstrate the close interrelations between the rDNA transcription and the maturation of the 100 kDa protein. We would like to postulate that the 100 kDa interacts with the rDNA, the RNA polymerase and the nascent transcript and that the elongation of transcription required the cleavage of the protein and the translocation of the maturation products on nascent RNA (15).

ACKNOWLEDGEMENTS

We are grateful to Professor J.P. Zalta for support en encouragement and I. Grummt for the gift of the pMr SP plasmid. This investigation was supported by grants from the Centre National de la Recherche Scientifique (3521) and Association pour le Développement de la Recherche sur le Cancer.

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