Metabolic heterogeneity of nuclear poly (A)-containing RNA in mouse liver

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SUMMARY

The analysis by the approach to equilibrium labeling method has shown that the poly(A)⁺ fraction of liver hnRNA is not a uniform class of molecules, but is comprised of two distinct subclasses with half-lives of 5 and 60 min, while the poly(A)⁻ hnRNA was metabolically homogeneous and turned over with a rather uniform half-life of 30 min. The results suggest that (a) poly(A) synthesis and addition is not limiting for the rate of hnRNA processing, and (b) there is a correlation between the kinetics of mRNA appearance in the cytoplasm and kinetic behavior of their possible nuclear precursors.

INTRODUCTION

The nuclei of all animal cells contain a population of rapidly turning over heterogeneous in size hnRNA with DNA-like base composition (1,2). Despite the data that the majority of this RNA is degraded within the nuclei, evidence has been accumulated that mRNA sequences are parts of hnRNA chains and therefore at least a fraction of hnRNAs could be considered as structural and metabolic precursors of mRNA (1,2). It has been suggested that mRNA sequences could be located on the 3'-end while the other part of the giant precursor could be degraded during processing (3). Discovery of poly(A) segments on the 3'-ends of some mRNAs and hnRNAs (1,4,5) has supported these ideas and raised the question of the mechanisms of processing.

One approach to study processing of hnRNA involves attempts to find structural and other similarities between hnRNA and mRNA: besides poly(A) both were found to contain methylated bases and nucleotides, rapidly hybridizing sequences, and self complementary regions (1). Recently a substantial extent

of sequence homology between mRNA and hnRNA was also found (6,7). The second approach is based on kinetic studies of synthesis and breakdown of hnRNA and attempts to relate them to the rate of appearance of mRNA in the cytoplasm. Darnell and coworkers have shown that poly(A) was added post-transcriptionally to hnRNA and that at least two different programs for hnRNA processing could exist; according to one of them poly(A) was added to giant transcripts immediately after synthesis; according to the second program the giant transcripts were cut to smaller segments prior to poly(A) addition (8).

Not all the mRNA molecules contain poly(A) (9-13). Initially it was assumed that only histone mRNA lacks poly(A) and due to this peculiarity rapidly enters polyribosomes without any noticeable lag (9). However, we have found that as much as 60-70% of liver mRNA did not contain poly(A) (10,11). Recently, poly(A)⁻ mRNA was found in other animal cells (12,13). Accordingly a more complicated problem, namely, the formation of poly(A)⁻ mRNA from its putative poly(A)⁻ hnRNA precursor, awaits its elucidation.

Newly-formed mRNA enters cytoplasmic polyribosomes after a definite delay termed lag-period (14). This time is usually considered necessary for processing and transport of mRNA into polyribosomes. Recently we demonstrated that in mouse liver there are three different poly(A)⁺ mRNA populations entering polyribosomes with definite but dissimilar delays of 10, 40, and 180 min respectively (15). If really the lag-period does reflect the processing time, then it can be expected that poly(A)⁺ hnRNA would contain at least three populations with different half-lives.

In the present work the study of the metabolism of hnRNA in mouse liver was carried out using kinetic approach to equilibrium labeling. Besides the relative content of poly(A)⁺ hnRNA, and the size of poly(A) segments of the poly(A)⁺ hnRNA were also determined.

MATERIALS AND METHODS

[2¹⁴-C] orotic acid with specific activity 35 mCi/mmmole and carrier-free ³²P orthophosphate were obtained from "Isotop",

USSR; [8-¹⁴C]adenine with specific activity 170 mCi/μmole was obtained from Radiochemical Center, Amersham, Great Britain. Cellulose was purchased from "Reanal" Budapest, Hungary, Poly(U) and poly(A) were obtained from Calbiochem, Los Angeles, USA; and DNase free of RNase - from "Worthington", USA. TI RNase was purchased from "Serva", Germany.

Buffer 1: 0.01 M NaCl, 0.02 M EDTA, 0.5% SDS (sodium dodecylsulfate), 0.01 M Tris-HCl, pH 7.6. Buffer 2: 0.01 M Tris-HCl, pH 9.0. Buffers were prepared in glass tridistilled water; pH values were adjusted at 20°.

Adult wild male mice (20 to 22 g) from our colony maintained on a standart diet were used. The animals were starved overnight before the experiment. Labeling with orotic acid, adenine, and [³²P]orthophosphate was accomplished by single i.p. injections of 20 μCi, 100 μCi and 1 mCi respectively and animals were killed at different time intervals after injections. The livers from 5-10 animals were pooled and placed on ice.

Liver nuclei were isolated according to Blobel and Potter (16) and fractionated into nucleoli and nucleoplasm for nucleic acid isolation by the method of Penman (17). The efficiency of the nucleolar separation was confirmed by the absence of pre-rRNA in nucleoplasmic RNA as revealed by sucrose gradient analysis. Polyribosomes were obtained as described elsewhere (15). Poly(A)-terminated RNAs were isolated directly from cell fractions by poly(U)-cellulose chromatography as described earlier (15) without prior RNA isolation. For this purpose nuclei, nucleoli and nucleoplasm were gently dissolved in Buffer 1 and heated for 1 min at 37°, than chilled to 20° and passed through a poly(U)-cellulose column preequilibrated with Buffer 1. The unadsorbed material contained the poly(A)⁻ RNA while the poly(A)⁺ was eluted with Buffer 2.

RNA was labeled with ³²P orthophosphate; poly(A)⁺ and poly(A)⁻ fractions were selected by poly(U)-cellulose chromatography, digested with the mixture of pancreatic and TI RNase and electrophoresed on polyacrylamide gels containing 8 M urea (18). Material of the two size classes was eluted (190-200 and 30-80 nucleotides) and the base composition of each determined

after alkaline hydrolysis according to Katz and Comb (19). Because the material of the size class ranging from 30 to 80 nucleotides contained not only oligoadenylate it was purified further by a second cycle of poly(U)-cellulose chromatography prior to base analysis.

This analysis showed that the poly(A)⁻ hnRNA fraction was free of any poly(A)₂₀₀ material and, therefore, was not contaminated by poly(A)⁺ hnRNA. The poly(A)⁺ hnRNA fraction contained both the poly(A)₂₀₀ tail and the oligo(A)₃₀₋₈₀ tract. Based on the inability of poly(U)-cellulose to bind long molecules containing only oligo(A) tracts the poly(A)⁺ hnRNA fraction was free of poly(A)⁻ hnRNA. Kinetic data also suggested that both poly(A)₂₀₀ and oligo(A)₃₀₋₈₀ segments belonged to the same hnRNA chains.

DNA and RNA were determined by the method of Schmidt-Tanhauser according to Blobel and Potter (20). The specific activity of the UTP of the cell soluble precursor pool and the correction of the RNA accumulation curve for the changing average specific radioactivity of the precursor pool were made as described earlier (15). Radioactivity of ¹⁴C-labeled samples was measured in Bray's solution using a liquid scintillation spectrometer MARK II (Nuclear Chicago, USA), and of ³²P-labeled samples by Cherenkov radiation.

RESULTS AND DISCUSSION

Poly(A)⁺ and poly(A)⁻ hnRNA in mouse liver nuclei

It was demonstrated that the nuclear poly(A)⁺ fraction of rapidly growing cells in culture contributed not more than 30% of steady state labeled hnRNA (1,7) and turned over with a half-life of 23 min (21).

To compare highly differentiated liver cells with rapidly dividing cells in culture we have measured the content of labeled poly(A)⁺ RNA in intact nuclei and in subnuclear fractions at different time intervals ranging from 5 min to 3.5 hr after a single injection of [2-¹⁴C]orotic acid. The results showed that (a) almost all the poly(A)⁺ RNA is found in the nucleoplasm either free or bound to chromatin; (b) the steady state amount of the poly(A)⁺ fraction comprised about 25% of

the total amount of nucleoplasmic RNA; and (c) in the course of fractionation of the nucleus there was no loss or tangible degradation of RNA. But the most important result obtained in these experiments showed that the fraction of poly(A)⁺ hnRNA strongly depended on the time of labeling: it comprised as much as 60% at a short labeling time (5 min) and then reached a plateau level at 1.5 hr - 3.5 hr. These results suggest that the majority of poly(A)⁺ hnRNA turned over faster than the poly(A)⁻ hnRNA fraction.

Kinetics of synthesis and degradation of poly(A)⁻ and poly(A)⁺ hnRNAs

To determine the half-lives of the two fractions of hnRNA, and to find out whether they are homogeneous or not in respect to turnover, we have obtained molar accumulation curves for UMP in both hnRNA fractions and subjected them to first-order decay analysis. The actual data of UMP accumulation in hnRNAs were corrected for the changing average specific radioactivity of the nuclear UTP pool by the method of Brandhorst and Humphreys (22) as described earlier (15). The results presented in Fig. 1 show that the poly(A)⁻ hnRNA may be characterized as class of molecules with an apparently uniform half-life of 30 min, while the poly(A)⁺ hnRNA is composed of at least two populations with half-lives of 5 and 60 min respectively. They contributed 75 and 25% respectively to the total poly(A)⁺ hnRNA.

To prove the metabolic heterogeneity of the hnRNA the labeled RNA was chased after actinomycin D treatment. Animals were labeled for 30 min with [¹⁴C]orotic acid in the presence of a low dose of actinomycin D (usually 4-5 µg per animal) to prevent synthesis of the pre-rRNA and RNA degradation was followed in the presence of a high dose of the same drug (20 µg per an animal) which completely prevented further RNA synthesis. First-order decay analysis of total hnRNA reveals two metabolic classes of the hnRNA with half-lives of 10 and 135 min (Fig. 2). They contribute 30 and 70% to the total labeled hnRNA respectively.

The results of this section clearly show that liver hnRNA

Fig. 1. Molar accumulation curves for uridine nucleotides in poly(A)⁺ and poly(A)⁻ hnRNA fractions of liver nuclei. Animals were injected with labeled orotic acid for various time intervals (5-180 min). The radioactivity in poly(A)⁺ and poly(A)⁻ hnRNA fractions computed per mg nuclear DNA was measured as a function of time. The actual data were corrected for the changing average specific radioactivity of the nuclear UTP pool according to Brandhorst and Humphreys (22) as described earlier (15). The curves (a,b) were computed using a computer program and the equation: $R = \sum_{i=1}^n R_{\infty} [1 - \exp(-\ln 2 x t / t_{1/2})]$, where R are the moles of labeled uridine in RNA at time t; R_{∞} - moles of labeled uridine in RNA under steady state conditions at equilibrium labeling; n - is the number of RNA metabolic classes; and $t_{1/2}$ is the RNA half-life. To determine $t_{1/2}$ the corrected data were subjected to first order decay analysis (c,d). a,c, poly(A)⁺hnRNA; b,d, poly(A)⁻ hnRNA.

is metabolically and structurally heterogeneous; there is a poly(A)⁻ fraction (70% of the total hnRNA) with a half-life of 30 min and two poly(A)⁺ fractions with half-lives 5 and 60 min which contribute 22 and 8% to the total hnRNA respectively. In the presence of actinomycin D the metabolism of hnRNA

Fig. 2. Kinetics of decay of hnRNA in the presence of actinomycin D. Animals were treated with a low dose of actinomycin D ($5 \mu\text{g}/20 \text{ g}$) and after 30 min were injected with labeled orotic acid and a chase was induced after 30 min labeling time with a high dose of actinomycin D ($20 \mu\text{g}/20 \text{ g}$). Total hnRNA was examined. Other methods were as described under legend to Fig.1.

is significantly altered; this suggests that normal processing of hnRNA is significantly altered; this suggests that normal processing of hnRNA depends on continuous synthesis of RNA and/or integrity of the chromatin structure.

CONCLUSIONS

In the experiments reported here we have made two new observations concerning the metabolism of hnRNA in liver nuclei. First, we have found that the poly(A)-terminated fraction which amounted to as much as 25% of the total nuclear hnRNA is comprised of at least two different metabolic classes which turned over with half-lives of 5 and 60 min, while the poly(A)⁻ hnRNA turned over with a rather uniform half-life of 30 min. Second, in the presence of actinomycin D in a dose, sufficient to inhibit by 98% the RNA synthesis, both fractions - the poly(A)⁺ hnRNA and the poly(A)⁻ hnRNA - appeared more stable, indicating a derangement of hnRNA processing.

The nature of the hnRNA is extremely complex and is not well elucidated as yet. There are data which indicate strongly

that the great majority of the single copy DNA is not made up of structural genes and is transcribed to yield hnRNA which is not a precursor to mRNA (23). But there is no doubt that the remainder of it is a structural precursor to mRNA, i.e. a true pre-mRNA. The pre-mRNA molecules may be processed within the nucleus as RNP particles in the nucleoplasm or else bound to chromatin to create mRNA in a form ready for exit to cytoplasm. Despite rapid progress in this field some important questions remain to be answered before the mechanism of mRNA biogenesis will be clarified. In particular, it is necessary to find out: (a) whether the structural and nonstructural single copy DNA transcripts are parts of the same hnRNA chains or they contribute to different chains; (b) how many mRNA chains are contained in one entire pre-mRNA molecule; (c) where is (are) the mRNA sequence(s) located within the pre-mRNA chain; and (d) what is the exact molecular mechanism of the specific cleavage of the pre-mRNA and what specific factors control the rate of this process?

The problem of the extent of transport of nuclear poly(A) to the cytoplasm is rather controversial. According to Perry et al. (24) a certain fraction of it is degraded within the nucleus and never reaches the cytoplasm, while Puckett et al. (25) showed that all the nuclear poly(A) was transported to the cytoplasm and entered polyribosomes. If we assume that in liver cells the situation is quite similar then all the poly(A) attached hnRNA molecules may be considered as pre-mRNA for poly(A)⁺ mRNAs, while poly(A)⁻ hnRNA might contain all the pre-mRNA for poly(A)⁻ mRNAs. The fractions of nuclear poly(A)⁺ hnRNA and the poly(A)⁺ mRNA in cytoplasm are consistent with this view: in the nucleus, as shown in this paper it was about 25% and in the cytoplasm 30% (10,18) of the total content of the respective RNA classes.

Using as a premise that the lag-period of mRNA entry into polyribosomes reflects the processing time of the putative hnRNA precursor, we should expect three poly(A)⁺ hnRNA fractions with half-lives not exceeding 10, 40 and 180 min, i.e. the lag-periods found earlier for the three poly(A)⁺ mRNA classes in liver cells (15). However, we have found only two poly(A)⁺

hnRNA fractions with half-lives of 5 and 60 min, while the third of the expected fractions with a higher half-life was not detected. We believe that this discrepancy is due to all the limitations imposed by the method of analysis. It is possible that the third fraction was not resolved by the present analysis and the fraction with the half-life of 60 min is actually a mixture of two fractions: one with a half-life lower than 60 min (perhaps 40 min) and the second with a higher half-life. If this interpretation is correct then our data are consistent with the view that the lag-period is a reflection of the processing time.

In HeLa cells mRNA appears in the polyribosomes after a lag-period of 20 to 30 min (14,25) and the hnRNA turned over with a uniform half-life of about 23 min (21). Recently Darnell and coworkers (26), using a specific inhibitor of hnRNA synthesis, attempted to define two classes of hnRNA in HeLa cells; their results suggest the possibility that at least some hnRNA molecules constitute precursors to mRNA while others are either not mRNA precursors or entered the cytoplasmic mRNA pool very slowly.

In HeLa cells the attachment of the poly(A) to hnRNA is a rapid process: it lasts for only 1-8 min, while the half-life of the majority of the hnRNA is about 20 min, suggesting that the synthesis and addition of the poly(A) are not the rate-limiting steps (8). The existence of two poly(A)-terminated hnRNA fractions with very distinct half-lives (5 and 60 min) also supports this idea. The derangement of the hnRNA processing by actinomycin D can be explained as follows. Recently Penman et al (7) have reported that nearly all the hnRNA is bound to chromatin being processed in this state. It is well known that actinomycin D binds to DNA and alters the structure of chromatin. Thus it may be expected that if processing does depend on the proper structure of chromatin actinomycin binding will derange this process.

Abbreviations: poly(A)⁺ and poly(A)⁻ RNAs = poly(A)-containing and poly(A)-lacking RNAs respectively; hnRNA = heterogeneous in size nuclear RNA with DNA-like base composition; AD = actinomycin D.

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