Age-Associated Changes in Initiation of Ribonucleic Acid Synthesis in Isolated Rat Liver Nuclei

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Initiation of RNA synthesis was studied in an attempt to determine a possible molecular mechanism for age-related biochemical and physiological changes. Initiation of RNA synthesis was determined by incorporation of [γ - 32 P]ATP and of [γ - 32 P]GTP into an acid-insoluble product by intact nuclei isolated from livers of Sprague-Dawley CD-strain rats of various ages. When the rats were grouped into young (0.75–9 months) and old (12–30 months) rats, a significant decrease ($P \leq 0.001$) in incorporation of initiating nucleotides was observed. The rat population was divided into five age groups (0.75–3 months, 4–9 months, 12–18 months, 19–23 months and 30 months) for further analysis of the effect of age on the initiation of RNA synthesis. Analysis of data from these groups indicated a significant trend for an age-related decrease in RNA-synthesis initiation (correlation coefficient = 0.94). Long-term hypophysectomy coupled with minimal hormone-replacement therapy was shown to have a significant effect on the reversal of the age-related decrease in initiation of RNA synthesis. It was observed that initiation of RNA synthesis in nuclei from 19-month-old rats, hypophysectomized at 12 months of age, was closest to that in 3-month-old intact rats and was not significantly different from that in liver nuclei of 0.75–9-month-old intact rats.

Age-related changes in RNA and protein synthesis have been observed in a number of biological systems (Mainwaring, 1968, 1969; Geary & Florini, 1972; Harchovec, 1972; Britton & Sherman, 1975; Buetow & Gandhi, 1975; Castle *et al.*, 1978; Bolla & Denckla, 1979). It has been suggested that the age-associated changes in RNA synthesis may occur at the level of initiation of RNA synthesis (Castle *et al.*, 1978; Bolla & Denckla, 1979) and may be influenced by an age-associated decrease in hormone induction of genome activity (Gusseck, 1972; Roth & Adelman, 1974; Adelman, 1975). It is apparent, however, that this age-related decrease in RNA synthesis is not due either to an increase in ribonuclease activity or to a decrease in available RNA polymerase (Britton *et al.*, 1972; Castle *et al.*, 1978; Bolla & Denckla, 1979). Bolla & Denckla (1979) have reported an age-associated decrease in the amount of chromatin-bound RNA polymerase and an increase in RNA polymerase free in rat liver

nuclei. Total rat liver nuclear RNA polymerase did not, however, change with age. Furthermore, these changes in chromatin-bound and free RNA polymerase activity were readily reversed in liver nuclei from long-term-hypophysectomized rats receiving replacement therapy with thyroxine plus tri-iodo-thyronine and somatotropin (Bolla & Denckla, 1979).

From the above observations, the studies reported in the present paper were designed to determine whether the number of sites available on chromatin for initiation of RNA synthesis decrease with age. In addition, the effects of long-term hypophysectomy and minimal hormone-replacement therapy (Bolla & Denckla, 1979) on initiation of RNA synthesis were investigated.

Materials and Methods

Experimental animals

Sprague-Dawley CD-strain rats, maintained under controlled temperature conditions on a 12h-light/12h-dark cycle, were used for these studies.

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For studies on the effect of hypophysectomy on initiation of RNA synthesis, rats of the experimental strain were hypophysectomized at 12 months of age by Zivic Miller Laboratories (Allison Park, PA, U.S.A.). These animals were maintained by the supplier as previously described (Bolla & Denckla, 1979). The rats were received at 18 months of age and were maintained on minimal hormone-replacement therapy as described previously (Scott *et al.*, 1979; Bolla & Denckla, 1979). Purina Rat Chow and water were available *ad libitum*. Any animals showing gross pathologies were discarded and not used in the experiments. To avoid diurnal variations all rats were killed by decapitation between 10:00 and 10:30 h.

Isolation of nuclei

Livers were excised rapidly and placed into ice-cold 50 mM-Tris/HCl (pH 7.4) that contained 250 mM-sucrose, 1 mM-MgCl₂ and 0.1 mM-2-mercaptoethanol. All procedures were at 4°C. The nuclei were isolated by centrifugation through dense sucrose as described previously (Widnell & Tata, 1964; Bolla *et al.*, 1977; Bolla & Denckla, 1979). The nuclear pellet was suspended in a small volume of homogenization buffer for use. DNA concentration was determined by the diphenylamine method of Burton (1956) and the nuclei were counted with a haemocytometer.

Initiation of RNA synthesis in isolated nuclei

Intact nuclei, rather than isolated chromatin, were used for the experiments presented. The initiation of RNA synthesis appears to be somewhat impaired in isolated chromatin (Reeder, 1973; Wilson *et al.*, 1975); however, genome expression appears to be maintained in intact nuclei (Reeder & Roeder, 1972; Gilboa & Aviv, 1976). GTP and ATP were used for these investigations, since the first nucleotide in an RNA molecule is usually ATP or GTP (Chambon, 1975).

RNA initiation was determined in a 200 µl reaction mixture, the basic composition of which was: 20 µmol of Tris/HCl (pH 7.9), 1 µmol of MgCl₂, 0.1 µmol of MnCl₂, 0.1 µmol of dithiothreitol, 1 µmol of phosphoenolpyruvate, 0.01 µmol of *S*-adenosylmethionine, 10 µg of pyruvate kinase and 0.12 µmol of CTP and UTP. In those experiments in which incorporation of both ATP and GTP was determined, the basic reaction mixture contained 0.03 µmol of both ATP and GTP and 4 µCi of both [γ -³²P]ATP and [γ -³²P]GTP (specific radioactivity of each 400 Ci/mmol; ICN, Irvine, CA, U.S.A.). ATP incorporation was determined in the basic reaction mixture containing 0.12 µmol of GTP, 0.03 µmol of ATP and 4 µCi of [γ -³²P]ATP. For studies of GTP incorporation, the reaction mixture contained, in addition to the basic components,

0.12 µmol of ATP, 0.03 µmol of GTP and 4 µCi of [γ -³²P]GTP. The reactions were initiated by the addition of 5×10^{12} nuclei from one of the various age groups. The reaction mixtures were incubated for 2 min at 27°C and the reaction was stopped by the addition of 10% (w/v) trichloroacetic acid containing 1% sodium pyrophosphate. The 2 min incubation time was chosen in order to avoid, at least in part, a large number of reinitiations and to minimize the effect of 5'-capping by which the initial [γ -³²P]phosphate residue could be removed from the newly initiated RNA and lead to a decreased estimation of RNA starts (Furuichi *et al.*, 1976; Stewart & Letham, 1977). This incubation period also lies within the linear portion of the rate curve (Fig. 1). Incorporation of the γ -³²P-labelled nucleotide was determined by Millipore-filter assay (Bolla *et al.*, 1977). Nuclei from a single liver were used for assays of the incorporation of each of the individual nucleotides.

Statistical analysis

Wide variations exist between chronological age and specific biological markers of maturity (Farris & Griffith, 1963). Grouping the rats by age provides an average representation of a specific trait for that age, thereby decreasing errors associated with variations between chronological age and biological maturity. Animals were grouped by age in one of two ways. In order to determine whether there was an overall age difference in the initiation of RNA synthesis, animals were grouped arbitrarily into younger (0.75–9 months of age) and older (12–30 months of age) rats, since the period of greatest fertility ends at 10 months (Farris & Griffith, 1963).

In order to determine if there was a continuous decline in the number of initiation sites with age, the rats were divided into five groups: a pre-pubescent group aged 0.75–3 months, a young adult group aged 4–9 months, a middle-aged group aged 12–18 months, an older adult group aged 19–23 months and a very old group aged 30 months. Means and standard errors of the means were calculated from standard formulae (Spiegel, 1961; Fisher & Yates, 1957), and statistical hypotheses were tested by using the two-tailed *t* test. Linear-regression fits and the corresponding correlation coefficients (*r*) were obtained by using standard formulae.

Results

Effect of age on RNA-synthesis initiation

After incubation to allow incorporation of [γ -³²P]phosphate, the nuclei were incubated for 10 min at 27°C in a 200 µl reaction mixture containing 20 µmol of Tris/HCl (pH 7.9), 1 µmol of MgCl₂ and 10 µg of deoxyribonuclease-free ribonuclease. Radioactivity remaining in trichloroacetic acid-precipit-

able material after this treatment was determined by Millipore-filter assay. It was observed that ribonuclease treatment of the nuclei removed $93.0 \pm 2.6\%$ (mean $\pm$ S.E.M., $n = 30$) of the incorporated radioactivity when compared with control nuclei incubated without ribonuclease.

The incorporation of both $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ into an acid-insoluble material by liver nuclei isolated from rats of various ages was determined statistically to be linear during the first 5–8 min of incubation, after which non-linearity was observed (Fig. 1). The rate of $\gamma\text{-}^{32}\text{P}$ -labelled nucleotide incorporation was not influenced by age (results not shown).

An overall age-related difference in initiation of RNA synthesis in liver nuclei was determined in rats grouped into younger and older animals. Table 1

Fig. 1. Time course of initiation of RNA synthesis in liver nuclei isolated from intact Sprague-Dawley CD-strain rats

Data represent incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ determined as described in the Materials and Methods section. The incorporation rates of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and of $[\gamma\text{-}^{32}\text{P}]\text{ATP} + [\gamma\text{-}^{32}\text{P}]\text{GTP}$ were identical with the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, and the results are not shown. Each data point represents the mean of ten determinations.

shows that younger (0.75–9 months of age) rats have significantly more total initiations with $\text{ATP} + \text{GTP}$ than do older (12–30 months of age) rats ($P \leq 0.001$). Incorporation of individual nucleotides was also observed to be significantly different between the two groups ($P \leq 0.002$ for GTP ; $P \leq 0.01$ for ATP). The greatest age-related change in RNA-synthesis initiation between the two groups of rats was observed in GTP -directed initiations. GTP -directed initiations were 4-fold greater in the young rats than in the older group.

By dividing the rats into five age groups as outlined in the Materials and Methods section, it was determined that differences between the younger and older animals (Table 1) occurred in a continuous manner. Thus there is a progressive decrease in the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, which was related to the age of the rat (Fig. 2). A similar age-associated decrease was observed for the incorporation of the individual nucleotides (Fig. 2). No significant change in initiation was, however, observed between the pre-pubescent and young adult groups. The greatest decrease in initiation of RNA synthesis occurred between 19 and 23 months of age and 30 months of age. This was apparent both for total initiation (by $\text{ATP} + \text{GTP}$) and for initiation by a specific nucleotide. The greatest effect of age on RNA-synthesis initiation was again observed to occur in GTP -directed initiation (Table 1, Fig. 2). The statistical fit of the regression line to the observed initiation values ($r = 0.79$) shown in Fig. 2 is not a good fit. This might suggest that, rather than a linear decline in GTP -directed initiations, there is a sharp decrease in GTP -directed initiations between 6 and 15 months of age, followed by a plateau until 30 months of age.

Effect of hypophysectomy on initiation of liver nuclear RNA synthesis

Studies were conducted to determine if long-term hypophysectomy coupled to minimal hormone-replacement therapy (Scott *et al.*, 1979), which has

Table 1. Initiation of RNA synthesis in rat liver nuclei isolated from young (0.75–9 months old) and old (12–30 months old) rats

Initiation was determined by the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into an acid-insoluble product during a 2 min incubation as described in the Materials and Methods section. Values are means $\pm$ S.E.M. for the numbers of animals in parentheses. All differences are significant at $P < 0.01$.

$\gamma\text{-}^{32}\text{P}$ -labelled nucleotide	$\gamma\text{-}^{32}\text{P}$ -labelled nucleotide incorporated (pmol/ 5×10^{12} nuclei)		<i>P</i>
	Young	Old	
ATP	63.2 $\pm$ 4.1 (43)	49.8 $\pm$ 3.0 (34)	0.01
GTP	16.5 $\pm$ 2.4 (40)	3.8 $\pm$ 0.6 (33)	0.0002
GTP + ATP	79.8 $\pm$ 5.8 (40)	55.1 $\pm$ 3.2 (32)	0.0001

Fig. 2. Correlation between initiation of RNA synthesis and age in liver nuclei from intact Sprague-Dawley CD-strain rats (○)

The rats were grouped into five groups according to developmental age. The number of animals used for each age group was as follows: 0.75–3 months old, 18; 4–9 months, 22; 12–18 months, 16; 19–23 months, 13; 30 months, 4; hypophysectomized, 4. Initiation was determined as described in the Materials and Methods section. Points are given as means $\pm$ S.E.M. $\blacktriangle$ represents the initiation of RNA synthesis in liver nuclei from Sprague-Dawley CD-strain rats hypophysectomized at 12 months of age and maintained until 19 months of age on a minimal hormone-replacement therapy (Bolla & Denckla, 1979) as compared with initiation in liver nuclei from intact animals. Correlation coefficients (r) are given in the Figure. Differences are significant at $P \leq 0.001$.

initiation. Liver nuclei isolated from 19-month-old rats that had been hypophysectomized at 12 months of age and maintained as previously described (Scott *et al.*, 1979) were assayed for RNA-synthesis initiation. Table 2 shows that incorporation of γ - ^{32}P -labelled nucleotides into an acid-insoluble product was significantly greater in nuclei from 19-month-old long-term-hypophysectomized rats receiving minimal hormone-replacement therapy than in nuclei from age-matched intact rats receiving the same hormone therapy. When compared with RNA synthesis in the rat groups in the overall age span, the γ - ^{32}P -labelled-nucleotide incorporation by liver nuclei from the long-term-hypophysectomized rats fits within the statistical range for the initiation of RNA synthesis in liver nuclei from rats aged 0.75–9 months (Fig. 2) and was closest to the value found in 3-month-old animals.

Discussion

It is apparent that there is an age-associated decline in rat liver nuclear RNA synthesis in both Sprague-Dawley and Fischer F344 rats (Bolla & Denckla, 1979; Castle *et al.*, 1978). However, the mechanism underlying this decrease is unclear. The data of Castle *et al.* (1978) and of Bolla & Denckla (1979) suggest that the change in RNA synthesis with age may involve changes in initiation of RNA synthesis. The trend towards an age-dependent decrease in initiation of liver nuclear RNA synthesis reported in the present paper would tend to support this possibility. The decrease in initiation which begins after 6–9 months of age in Sprague-Dawley rats appears to be continuous over the span of ages investigated and corresponds to the progressive decrease in RNA synthesis observed after 6 months of age (Bolla & Denckla, 1979).

It is unlikely that the decrease in RNA-synthesis initiation is due to a restriction of available RNA polymerase in the nuclei, to increased nuclear ribonuclease activity, to changes in endogenous nucleotide pools or to nuclear metabolism of nucleotides. These parameters have not been shown to change appreciably with age in isolated rat liver nuclei (Castle *et al.*, 1978; Bolla & Denckla, 1979). An age-associated decrease in the amount of nuclear RNA polymerase bound to chromatin and an age-associated increase in free nuclear RNA polymerase activity has, however, been reported (Bolla & Denckla, 1979). This suggests that with increasing age there is a decrease in available initiation sites on nuclear chromatin.

The greatest effect of age on the initiation of RNA synthesis was seen in GTP-directed initiation. This observation might suggest that 5'-capping (Furuichi *et al.*, 1976) of RNA molecules synthesized *de novo* increases with age such that through loss of the

been shown to effect a reversal of the age-related decrease in total nuclear RNA synthesis (Bolla & Denckla, 1979), manifests its effect at the level of

Table 2. *Initiation of RNA synthesis in liver nuclei isolated from 19-month-old intact rats and long-term-hypophysectomized rats*

The rats were hypophysectomized at 12 months of age and maintained as described by Scott *et al.* (1979) and by Bolla & Denckla (1979) until they were 19 months of age. Initiation was determined as described in the Materials and Methods section. Values reported are means $\pm$ S.E.M.; the number of animals used in each experiment is given as (n) for each experimental group.

γ - ³² P-labelled nucleotide	γ - ³² P-labelled nucleotide incorporated (pmol/5 $\times$ 10 ¹² nuclei)		P
	Hypophysectomized (n = 4)	Intact (n = 9)	
ATP	118.0 $\pm$ 50.9	115.0 $\pm$ 6.7	0.01
GTP	36.7 $\pm$ 8.2	6.61 $\pm$ 1.9	0.002
ATP + GTP	224.7 $\pm$ 53.5	121.6 $\pm$ 7.2	0.01

5'-terminal [γ -³²P]phosphoryl residue a number of initiation events might not be recorded by the assay method used. The apparent linearity of [γ -³²P]GTP incorporation during the initial 10 min of the initiation assay in all ages of rats examined might suggest, however, that 5'-capping was not a major influencing factor in the 2 min incubations used for the assays. This cannot conclusively be determined without analysis of the 5'-terminal residue of initiated polynucleotides (Cedar & Felsenfeld, 1973; Gilboa *et al.*, 1977). The possibility that GTP-directed initiations might be more greatly affected by age than ATP-directed initiations is interesting; however, additional evidence is necessary to ascertain this observation.

Long-term hypophysectomy coupled with minimum hormone-replacement therapy has been shown to result in a reversal of the age-related decline in several physiological parameters (Denckla, 1974; Bilder & Denckla, 1976; Parker *et al.*, 1978; Scott *et al.*, 1979). Similarly, initial studies on the effect of this treatment on liver nuclear RNA synthesis suggests a reversal of the age-associated decrease in this biochemical parameter (Bolla & Denckla, 1979). On the basis of the observation that the chromatin-bound and free liver nuclear RNA polymerase activity in 22-month-old rats hypophysectomized at 12 months of age and maintained on hormone-replacement therapy was more comparable with that of 6-month-old rats than with that of age-matched intact rats, Bolla & Denckla (1979) suggested that the effect of this treatment on RNA synthesis was mediated through increased initiation of RNA synthesis. The present observation that initiation of liver nuclear RNA synthesis in 19-month-old rats hypophysectomized at 12 months of age was more comparable with initiation in 0.75–9-month-old rats than with intact age-matched rats and was closest to that in 3-month-old rats would tend to support this hypothesis. It cannot be determined from the present studies, however, whether

the increased initiation observed in liver nuclei from the long-term-hypophysectomized rats simply represents increased re-initiation or if it represents an actual increase in single initiation events. This question must be answered by determining size classes of RNA initiated and elongated in these nuclei in the absence of re-initiation as compared with control nuclei.

In conclusion, a trend towards an age-associated decrease in the initiation of liver nuclear RNA synthesis has been observed which might explain the mechanism for the reported decreased liver nuclear RNA synthesis in older rats (Castle *et al.*, 1978; Bolla & Denckla, 1979). Additionally, this decrease can apparently be reversed by long-term hypophysectomy and minimal hormone-replacement therapy with tri-iodothyronine + thyroxine and somatotropin.

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