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High Concentration of RNA Polymerase I is Responsible for the High Rate of Nucleolar Transcription

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When isolated rat liver nuclei and nucleoli are compared for RNA synthesis *in vitro*, the rate of nucleolar RNA synthesis is found to be more than 10 times higher. In order to understand this high rate of nucleolar transcription, DNA from both nuclear and nucleolar fractions was isolated and compared for the ability to direct RNA synthesis with homologous RNA polymerases. No difference between these two templates is evident. On the other hand, when the total nuclear and nucleolar RNA polymerases are isolated and compared on a per-unit-weight-of-DNA basis, it becomes clear that the nucleolus has a 10-fold higher RNA polymerase concentration than the nucleus. This result suggests that RNA polymerase I concentration rather than the nucleolar DNA template efficiency is responsible for the observed high rate of nucleolar transcription under the normal steady-state condition.

The nucleolus, a subnuclear organelle rich in ribonucleoprotein, is the site of precursor-rRNA synthesis (Perry, 1962; Brown & Gurdon, 1964; Ritossa & Spiegelman, 1965). Pulse-labelling studies have shown that nucleolar RNA synthesis accounts for 40–50% of the total nuclear RNA synthesis (Warner *et al.*, 1966; Darnell, 1968; Soeiro *et al.*, 1968). Since nucleolar DNA is only 4–5% of the total nuclear DNA content (Muramatsu *et al.*, 1963; Steele, 1968; Mohan *et al*, 1969; Busch & Smetana, 1970), it is clear that the rate of nucleolar transcription is 10 times greater than the overall rate of nuclear transcription. The reason for this great difference in transcription rate has not been previously explored.

When analysing the various elements that may be involved in the regulation of gene expression in eukaryotes, e.g. the DNA template, the chromatin and the RNA polymerase, it was found the only clear difference between the nuclei and the nucleolus that can explain the high rate of nucleolar transcription is the fact that, on a per-unit-weight-of-DNA basis, the nucleolus has a 10-fold higher RNA polymerase concentration than the nucleus.

Experimental

Isolation of rat hepatic nuclear and nucleolar fractions

Male Sprague–Dawley rats weighing about 200g were used. Rat liver nuclear and nucleolar fractions were isolated by the hyperosmotic-sucrose method, as described previously (Yu, 1974, 1975*a*). Rats were stunned by a blow on the head and killed by cervical dislocation. The livers were excised after perfusion with cold 0.25M-sucrose/3.3mM-$CaCl_2$ solution. All subsequent operations were conducted at 0–4°C. The livers were weighed and then homogenized in 2vol. of 2.3M-sucrose/3.3mM-$CaCl_2$ in a glass homogenizer loosely fitted with a Teflon pestle. The homogenate was filtered through four layers of cheese-cloth and was finally adjusted by further mixing with 8vol. more of the 2.3M-sucrose/3.3mM-$CaCl_2$ solution. The homogenate was then centrifuged at 40000*g* for 1h in a Spinco 30 rotor. The resultant nuclear pellet was suspended in 0.34M-sucrose (1ml/g of original liver). For nucleolar isolation, 12ml of the above nuclear suspension was sonicated with a Biosonik IV sonicator, equipped with a fine probe, until virtually all nuclei were broken (nuclear disruption was monitored by phase microscopy). The sonicated preparation was layered over 12ml of 0.88M-sucrose and centrifuged at 2000*g* for 20min at 0°C to sediment the nucleoli. The nucleolar pellet was also suspended in 0.34M-sucrose (1ml/6g of original liver).

Separation and solubilization of nuclear free and engaged RNA polymerases

The procedures are essentially the same as reported previously (Yu, 1975*a*). The nuclear free

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RNA polymerase was separated from the engaged enzyme by gentle homogenization of the isolated nuclei in 0.34M-sucrose, followed by centrifugation for 10min at 3000*g*. The nuclear pellet retaining all the engaged enzyme was suspended in 0.01M-Tris/HCl buffer, pH7.9, containing 1M-sucrose, 5mM-$MgCl_2$ and 20mM-2-mercaptoethanol and solubilized as described by Roeder & Rutter (1970).

Assay of RNA polymerase activity

As described previously (Yu, 1975*a*, 1977), the nuclear and nucleolar RNA polymerase activities (Fig. 1, solid lines) were assayed *in vitro* with a standard assay medium (volume 0.5ml) containing 0.1M-Tris/HCl (pH7.9 at 23°C), 2mM-$MnCl_2$, 20mM-2-mercaptoethanol, 65mM-$(NH_4)_2SO_4$ and 0.2mM each of unlabelled ATP, GTP, UTP and CTP with 0.1μCi of [8-^{14}C]GTP (New England Nuclear, Boston, MA, U.S.A.; specific radioactivity 45.7Ci/mol). The reaction was initiated by the addition of 0.1ml of the nuclear or nucleolar suspension and the mixture was incubated at 37°C for various times as indicated, with shaking. When purified nuclear or nucleolar DNA was used as template to support RNA synthesis with various RNA polymerase preparations (Fig. 1 and Table 1), 0.2ml of the standard assay medium mentioned above was used, and 0.04mM unlabelled GTP with 2μCi of [8-^{3}H]GTP (New England Nuclear; specific radioactivity 10.8Ci/mmol) were used instead. Purified nuclear or nucleolar DNA (3μg) in 50μl of water was added to the assay medium and the reaction was initiated by the addition of 50μl of the enzyme solution in buffer A [0.05M-Tris/HCl (pH7.9 at 23°C), 25% (v/v) glycerol, 5mM-$MgCl_2$, 0.1mM-EDTA and 20mM-2-mercaptoethanol] containing 50mM-$(NH_4)_2SO_4$. The incubation was carried out at 37°C for 20min (Table 1) or for various times as indicated (Fig. 1, broken lines). For the assay of the individual RNA polymerase species obtained from DEAE-Sephadex column chromatography (Yu, 1975*b*, 1977), 0.1ml of the enzyme solution in buffer A containing 50mM-$(NH_4)_2SO_4$ was added to the 0.2ml standard assay medium containing 2.5μg of poly(dI-dC) (Boehringer Mannheim, Indianapolis, IN, U.S.A.) and incubated at 37°C for 20min with shaking (Table 2). The reaction was stopped by transfer of the reaction tubes to chipped ice, followed by immediate addition of 5ml of cold (4°C) 10% (w/v) trichloroacetic acid containing 1% sodium pyrophosphate. The acid-insoluble material was collected on Whatman GF/C filters and washed with 7 x 5ml of cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and once with 5ml of 60% (v/v) ethanol. The filters were air-dried in liquid-scintillation vials and the radioactivity was counted in 10ml of Bray's (1960) solution. The specific activity of RNA polymerase was expressed as pmol of [^{14}C]GMP or [^{3}H]GMP incorporated/mg of DNA; DNA concentrations were determined as described by Burton (1968).

Isolation of rat liver nuclear and nucleolar DNA

The procedure of Okuhara (1970) was used.

Results and Discussion

Fig. 1 (solid lines) compares the kinetics of rat liver nuclear and nucleolar RNA synthesis *in vitro*. It is clear, on a per-mg-of-DNA basis, that the rate for nucleolar RNA synthesis is more than 10 times greater than the nuclear RNA synthesis. This result is in good agreement with earlier observations (Yu & Grunberger, 1976; Yu, 1977). In order to understand the reason for this high rate of nucleolar RNA synthesis, the following possibilities have been considered. (*a*) The nucleolar DNA is intrinsically a much better template for transcription than the rest of the nuclear DNA. (*b*) The nucleolar chromatin is organized in such a way that makes the nucleolar DNA a much better template for transcription. (*c*) The enzyme, RNA polymerase I, that transcribes the nucleolar DNA template exists in a much higher

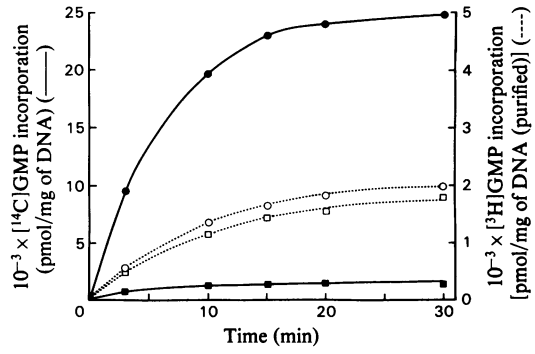

Fig. 1. *Kinetics of nuclear, nucleolar, nuclear-DNA- and nucleolar-DNA-directed RNA synthesis in vitro*

Male Sprague–Dawley rats weighing about 200g were used. Rat liver nuclear and nucleolar fractions were isolated by the hyperosmotic-sucrose method, as described previously (Yu, 1975*a*, 1977). The nuclear and nucleolar DNA were isolated by the method of Okuhara (1970). Nuclear and nucleolar RNA synthesis was carried out under the assay conditions described by Yu (1975*b*, 1977). The nuclear-DNA- and nucleolar-DNA-directed RNA syntheses were assayed with the isolated rat liver total nuclear engaged RNA polymerase (Yu, 1975*a*). For detailed assay conditions, see the Experimental section. Values given are means of two separate experiments. ■——■, Nuclei; ●——●, nucleoli; □----□, nuclear DNA; O----O, nucleolar DNA.

concentration on a per-unit-weight-of-DNA basis than the nucleoplasmic RNA polymerases, i.e. RNA polymerases II and III.

As reported previously (Yu, 1975*a*), there are great differences in template efficiency for various DNA species, so it is reasonable to consider that the nucleolar DNA might be intrinsically a better template. However, as shown also in Fig. 1 (broken lines), when purified total nuclear and nucleolar DNA were used as templates for transcription with solubilized homologous rat liver nuclear engaged RNA polymerase, no difference in efficiency for these two templates could be detected. The same conclusion was reached when various concentrations of the above-mentioned DNA templates were tested (results not shown).

However, since solubilized nuclear engaged RNA polymerase is composed of a mixture of RNA polymerases, roughly 50% RNA polymerase I, 35% RNA polymerase II and 15% RNA polymerase III (Yu, 1975*b*; Yu & Grunberger, 1976; Yu, 1977), this test may not necessarily reflect the selective affinity of the individual RNA polymerase species toward its native template. In order to eliminate this possibility, the nucleolar and nuclear DNA templates were tested with various RNA polymerase preparations, and the results are shown in Table 1. It is clear from these data that, no matter what RNA polymerase source was used, the nucleolar DNA was not a better template than the total nuclear DNA. Thus the high rate of nucleolar transcription is due to something else.

Next, the possibility whether there is any difference in transcriptional efficiency between the nuclear and nucleolar chromatin was investigated. However, it was found that the isolated chromatin preparations were extremely poor templates for transcription *in vitro* with homologous RNA polymerase. [In fact, this is a well-known phenomenon since the early work of Bonner and co-workers (Dahmus & Bonner, 1970) and of Paul & Gilmour (1968)]. Because of the realization that bacterial RNA polymerase does not transcribe mammalian chromatin properly (Keshgegian & Furth, 1972; Zasloff & Felsenfeld, 1977; Fodor & Doty, 1977) and no meaningful result could be obtained if *Escherichia coli* RNA polymerase was used, the heterologous system was therefore not tested. Still, from the few initial trial experiments with the homologous RNA polymerase preparations, it did not appear that there were any obvious differences.

Finally, the possibility was explored that the high rate of nucleolar transcription might be a reflection of the high concentration of RNA polymerase I in the nucleolus. To carry out this type of study, it is necessary to isolate the total RNA polymerase quantitatively and then separate them effectively into individual RNA polymerase species. As pointed out previously (Yu, 1975*a*), the conventional method of sonication in high salt to solubilize RNA polymerase inevitably destroys the free RNA polymerase fraction, which accounts for almost 50% of the total RNA polymerase population in rat liver nuclei (Yu, 1975*a*). An improved method was developed (Yu, 1975*a*) and used in this study. After the total nuclear free and engaged RNA polymerases were separately extracted (Yu, 1975*a*), the three individual RNA polymerase species were resolved by using DEAE-Sephadex column chromatography (Yu, 1975*b*; Yu & Grunberger, 1976; Yu, 1977). The column recovery was essentially 100% (Yu, 1977). Table 2 shows the normal quantitative distribution of various RNA polymerase species per g of liver. There are several points that should be discussed. (*a*) In agreement with earlier reports (Yu, 1974, 1975*a*), the total nuclear free RNA polymerase is a significant fraction (40–50%) of the total nuclear RNA polymerase population. The importance of this fraction of RNA polymerase in the elucidation of the mechanism of hormone action, chemical carcinogenesis, cell growth and differentiation is well illustrated in several reports from our (Yu & Feigelson, 1971; Yu, 1975*b*; Yu & Grunberger, 1976; Yu, 1977) and other laboratories (Adams & Goodman, 1976; Fuhrman & Gill, 1976; Hentschel & Tata, 1977; Anderson *et al.*, 1977; Zonchedder *et al.*, 1977; Kellas *et al.*, 1977). (*b*) The total activity (free plus engaged) of RNA polymerases I, II and III each accounts for about one-third of the total nuclear RNA polymerase activity. (*c*) There are great variations in the relative distributions between the free and the engaged forms

Table 1. *Comparison of the template efficiency between rat liver nuclear and nucleolar DNA in directing RNA synthesis with various homologous RNA polymerases*

Rat liver nuclear and nucleolar DNA were isolated by the method described by Okuhara (1970). The efficiency of the DNA templates (3 μg) was assayed with various solubilized homologous RNA polymerase preparations in a 0.2 ml standard assay medium containing 2 μCi of [8-^{3}H]GTP. The incubation was carried out at 37°C for 20 min (see the Experimental section for details). Values are given as means ± S.E.M. for three separate experiments.

	RNA polymerase activity (pmol of [^{3}H]GMP incorporated/mg of DNA)		
RNA polymerase	Nuclear DNA (*a*)	Nucleolar DNA (*b*)	100 × *a*/*b* (%)
Free enzyme	1168 ± 189	963 ± 89	121
Engaged enzyme	1722 ± 143	2008 ± 262	86
I	141 ± 16	121 ± 14	117
II	114 ± 13	88 ± 15	129

Table 2. *Normal distribution of the free and engaged enzymes together with each of the RNA polymerase species in rat liver nuclei*

The free and engaged RNA polymerases were first sequentially extracted from the nuclei (Yu, 1975*a*). The individual RNA polymerase species were separated by DEAE-Sephadex columns (Yu, 1975*b*, 1977). The peak fractions were pooled and assayed as in Table 1, except that 0.1 μCi of [8-^{14}C]GTP and 2.5 μg of poly(dI-dC) were used for these asays. Values are given as means ± S.E.M. derived from six separate experiments.

	RNA polymerase activity (pmol of [^{14}C]GMP incorporated/g of liver)									
	I		II		IIIa		IIIb		Total	
Enzyme fraction	(pmol/g)	(%)	(pmol/g)	(%)	(pmol/g)	(%)	(pmol/g)	(%)	(pmol/g)	(%)
Total	1397 ± 118	100	1140 ± 110	100	502 ± 38	100	495 ± 42	100	3534	100
Free	304 ± 36	22	532 ± 67	47	277 ± 45	55	333 ± 38	67	1446	41
Engaged	1093 ± 97	78	608 ± 62	53	225 ± 29	45	162 ± 25	33	2088	59

of RNA polymerases within each RNA polymerase species. Thus, for RNA polymerase I, 78% of it exists as engaged enzyme, and only 22% in the form of free enzyme. On the other hand, for RNA polymerase III, especially IIIb, about two-thirds of its exists as free enzyme, and only one-third as engaged enzyme. For RNA polymerase II, the free and the engaged enzymes are evenly distributed.

Since nucleolar DNA accounts for only 4–5% of the total nuclear DNA (Muramatsu *et al.*, 1963; Mohan, *et al.*, 1969; Busch & Smetana, 1970), and in view of the finding (Table 2) that the total RNA polymerase I represents very nearly one-third of the total nuclear RNA polymerase population, it can be easily calculated on a per-unit-of-DNA basis that the nucleolar DNA has a more than 10-fold higher RNA polymerase concentration than the overall nuclear DNA. The calculated value is even higher if only the engaged RNA polymerases alone are considered. In such a case, the engaged RNA polymerase I represents close to 50% of the total nuclear engaged RNA polymerases, and the RNA polymerase density per unit of nucleolar DNA is thus almost 20 times that of the overall nuclear DNA. In fact, this high rate of nucleolar RNA synthesis is occasionally observed (Yu, 1977).

It is clear from the data presented above that the most logical conclusion to explain the order-of-magnitude higher rate of nucleolar RNA transcription is the greater than 10-fold higher concentration of RNA polymerase I in the nucleolus. It is not clear, however, why there is such a tremendous difference in the relative distribution between the free and engaged forms of the various species of RNA polymerase. This question apparently cannot be answered at present until we know more about the basic difference(s) between the free and the engaged RNA polymerases.

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