Modification of the Template Capacity of Liver Chromatin for Form-B Ribonucleic Acid Polymerase by Food Intake in Rats under Controlled Feeding Schedules

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(Received 3 August 1974)

1. Nuclei from liver of rats accustomed to eating during the first 8 h of a daily 12 h dark period demonstrate an increased capacity to synthesize RNA 6 h after the beginning of the feeding period. 2. This increase is accompanied by a higher yield of extractable form-B DNA-dependent RNA polymerase activity. 3. The endogenous RNA polymerase activity associated with nuclear chromatin is also stimulated by food intake. Both purified and chromatin-associated form-B enzyme activities exhibit different ionic strength requirements after food intake. 4. The sensitivity of exogenous (added) form-B enzyme to changes in ionic strength changes after feeding when chromatin is used as template. 5. Chromatin extracted from the liver of fed rats is a better template for form-B enzyme than chromatin extracted from starved rats.

The control of the feeding schedules of laboratory animals has been shown to have an important effect on enzymic activities of the liver (Potter *et al.*, 1968; Barbiroli & Potter, 1971; Hopkins *et al.*, 1973*b*). Many enzymic activities fluctuate daily and depend on the feeding conditions or are induced by the food intake (Potter, 1970; Scott & Potter, 1970; Gregory *et al.*, 1972; Hopkins *et al.*, 1973*a*). It has been found in this laboratory that the DNA-dependent RNA synthesis measured in purified nuclei (Barbiroli *et al.*, 1973*b*) is modulated by the food intake, and that these daily fluctuations of RNA synthesis also seem to involve the chromatin DNA template (Barbiroli *et al.*, 1973*a*).

Clark & Felsenfeld (1971) and Itzhaki (1970) have shown that as much as 35–50% of chromatin DNA may not be masked by chromosomal proteins. Conversely, only 5–20% of the total DNA of chromatin appears to be available for transcription by the bacterial RNA polymerase (Bonner *et al.*, 1968; Paul & Gilmour, 1968; Tan & Miyagi, 1970). The recent demonstration that mammalian nuclei contain several different species of DNA-dependent RNA polymerase which may be distinguished from one another by their chromatographic behaviour (Roeder & Rutter, 1969), their subunit composition (Mandel & Chambon, 1971) and their sensitivity to the toxin α -amanitin (Mandel & Chambon, 1971) illustrates the complexity of the transcriptional process. Further, the studies done so far to define some characteristics of RNA polymerases and chromatin have been on the livers of rats maintained on an *ad libitum* feeding schedule (Butterworth *et al.*, 1971).

In the present study we have examined the effect of food intake on RNA synthesis in the rat liver. Three aspects have been particularly investigated: first, the amounts of form-A and form-B RNA polymerase activities extractable from liver nuclei at two different times of day corresponding to low and high rates of RNA synthesis, i.e. starving and feeding conditions; secondly, changes in the activity of RNA polymerase associated with chromatin, and finally changes in the template activity of chromatin for homologous form-B RNA polymerase.

Materials and Methods

[5-³H]Uridine 5'-triphosphate (sp. radioactivity 22 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.); unlabelled nucleotide triphosphates were from Boehringer Mannheim G.m.b.H. (Mannheim, Germany); dithiothreitol was from Sigma Chemical Co. Ltd. (St. Louis, Mo., U.S.A.); DEAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden); α -amanitin was from Boehringer und Sohne (Ingelheim, Germany) and rifampicin AF/0-13 from Biochemia, S.r.l. (Milano, Italy).

Animals

Seven-week-old male albino rats of the Wistar strain weighing 200–220 g were used in these experiments. At 21 days of age they were housed usually two per cage in an air-conditioned windowless room lighted from 21:00 to 09:00 h. Food was supplied just before the lights were switched off, and was

removed 8 h later according to the feeding schedule developed by Potter *et al.* (1968) and Potter (1970). Water was supplied *ad libitum*.

Nuclei

Rats were killed by decapitation, the livers were quickly removed and homogenized in 0.32M-sucrose containing 1mM-MgCl₂ and 1mM-dithiothreitol.

After sedimentation at 1000g for 20min, the nuclei were purified further by the method of Widnell & Tata (1964) by sedimentation in 2.3M-sucrose, 1mM-MgCl₂ and 1mM-dithiothreitol at 50000g in a Spinco centrifuge for 60min.

Chromatin preparation

Chromatin was isolated from purified nuclei obtained from 14g of liver by the technique of Butterworth *et al.* (1971). Nuclei were suspended in 14ml of 0.025M-NaCl containing 8mM-EDTA, pH 8.0, and centrifuged at 900g for 10min. The nuclear sediment was washed again in the same buffer and centrifuged.

The resulting sediment was homogenized in 28ml of 0.01M-Tris-HCl buffer, pH 8.0, stirred at 0°C for 60min, the suspension rehomogenized and centrifuged at 12000g for 15min. The sediment obtained was suspended in 14ml of 1.7M-sucrose containing 0.01M-Tris-HCl buffer, pH 7.4, by homogenization, and layered over 10ml of 1.7M-sucrose containing 0.01M-Tris-HCl, pH 7.4, and centrifuged at 90000g for 180min in the SW 25.1 rotor in a Spinco L65 2B centrifuge. The clear sediments were resuspended by mild homogenization in 14ml of 1mM-Tris-HCl, pH 8.0. The chromatin was assayed for DNA by the method of Burton (1956), and for protein content by the procedure of Lowry *et al.* (1951); it had a DNA/protein ratio of 1:1.58 (average of all determinations performed) (Barbiroli *et al.*, 1973a). The chromatin was used immediately to study the template activity and endogenous RNA polymerase activity.

DNA preparation

DNA was prepared from purified rat liver chromatin by the addition to chromatin of CsCl to a final concentration of 4.0M (Butterworth *et al.*, 1971). The mixture was then centrifuged at 170000g for 30h in the 65 Ti rotor of the Spinco L2 65B centrifuge. The sediments were dissolved in 0.01M-Tris-HCl, pH 8.0, containing 0.5mM-EDTA, and the resulting solution was dialysed against 1000vol. of the same buffer for 16h with two changes. The resulting solution was diluted to a final concentration of DNA of 2mg/ml and stored at -15°C.

Preparation of rat liver form-A and form-B RNA polymerase

Nuclei purified from 100g of rat liver were suspended in 40ml of 1.0M-sucrose - 5mM-MgCl₂ - 1mM-dithiothreitol - 10mM-Tris-HCl, pH 8.0, and sonicated at full power with a Branson medium probe for 1min in 10s bursts after the addition of 2.9ml of 4M-(NH₄)₂SO₄. The temperature was maintained at 0°C and the subsequent steps were performed at 3°C. The sonicate was poured into 88ml of 0.05M-TGMED medium [Tris-HCl (pH 8) - 25% (v/v) glycerol - 5mM-MgCl₂ - 0.1mM-EDTA - 0.5mM-dithiothreitol] and cleared by centrifugation at 30000g for 30min. The enzymes were precipitated by slowly adding 0.42g of (NH₄)₂SO₄ per ml of supernatant with stirring under N₂ and sedimented at 30000g for 20min. The sediment was dissolved in 20ml of 0.05M-(NH₄)₂SO₄ in TGMED buffer and dialysed against this buffer overnight. After sedimenting any insoluble material by centrifugation at 30000g for 30min, the supernatant was loaded on to a column (30cm x 2.5cm) of DEAE-Sephadex (A-25) previously equilibrated with 0.05M-(NH₄)₂SO₄ in TGMED buffer. The column was subsequently washed with the same buffer until the absorbance at 280nm of the eluate reached a plateau. A 250ml linear gradient of 0.05-0.5M-(NH₄)₂SO₄ in TGMED buffer was applied, followed by a further 100ml of 0.5M-(NH₄)₂SO₄ in TGMED buffer. The flow rate was maintained at 80ml/h. Fractions (2ml) were collected, and samples were assayed for RNA polymerase. Active fractions were combined and stored at -70°C.

Assays of DNA-dependent RNA polymerase activities

(1) *In purified nuclei.* The two DNA-dependent RNA polymerase activities were assayed by the α -amanitin differential assay developed by Novello & Stirpe (1970). Purified nuclei (90-100 μ g of DNA) were incubated for 15min in the reaction mixture, final volume 0.5ml. For RNA polymerase A, each assay contained: 50 μ mol of Tris-HCl buffer, pH 8.0; 2 μ mol of MgCl₂; 7 μ mol of mercaptoethanol; 3 μ mol of NaF; 0.3 μ mol each of CTP, GTP and ATP; 0.02 μ mol of [³H]UTP (55d.p.m./pmol); 1 μ g of α -amanitin. For RNA polymerase B, assays contained 50 μ mol of Tris-HCl buffer, pH 8.0; 2 μ mol of MnCl₂; 0.14mmol of (NH₄)₂SO₄; 0.9 μ mol each of CTP, GTP and ATP; 0.060 μ mol of [³H]UTP (22d.p.m./pmol) and 1 μ g of α -amanitin (when present). The reaction was stopped by adding 1mg of serum albumin and 5ml of 0.5M-HClO₄.

(2) *With rat liver chromatin or DNA as template.* All assays were performed by the method of Cox *et al.* (1973) and the reaction mixtures contained, in a final volume of 0.25ml: 6.25mmol of Tris-HCl,

pH 8.0, 0.25 μmol of MnCl_2 , 0.125 μmol of dithiothreitol, 0.075 μmol each of GTP, CTP and ATP and 12.5 nmol of [^3H]UTP (44 d.p.m./pmol).

Reaction mixtures were incubated at 37°C. RNA synthesis was terminated by addition of 1 ml of ice-cold 10% (w/v) trichloroacetic acid–3% (w/v) $\text{Na}_4\text{P}_2\text{O}_7$. The precipitate was collected on Whatman GF/C filters and the radioactivity measured in a Nuclear–Chicago ISOCAP 300 liquid-scintillation spectrometer with an efficiency of about 40–45%.

Release of proteins from chromatin

To chromatin containing 0.6 mg of DNA and 0.95 mg of protein/ml was added an equal volume of the relevant salt solution required to obtain specific final salt concentrations. These solutions were centrifuged at 140 000g for 20 h at 2°C and the resulting supernatants were dialysed against two changes of deionized water at 4°C and finally freeze-dried. Protein was determined by the procedure of Lowry *et al.* (1951) on the centrifuged chromatin and supernatant fractions.

Results

Definition of the experimental condition

Before performing a detailed analysis of the components involved in transcription we evaluated the rates of RNA synthesis in whole nuclei purified from the livers of rats maintained on controlled feeding schedules at two different times of day, by measuring the DNA-dependent incorporation of [^3H]UTP into an acid-insoluble product. Measurements were made on liver nuclei from rats killed at 09:00h and 15:00h, the two times of day at which previous experiments (Barbiroli *et al.*, 1973b) had shown low and high activity respectively. The low activity comes after 16 h of starvation and the high activity is the result of re-feeding from 09:00h in the rats maintained on the controlled feeding schedules developed by Potter *et al.* (1968).

Table 1 indicates that at the two selected times of day the incorporation of labelled UTP into RNA is affected by food intake. In fact rats killed at 09:00h, having been starved overnight since 17:00h, showed a low rate of RNA synthesis for both form-A and form-B enzymes, whereas rats killed at 15:00h, having been allowed food since 09:00h, showed large increases in both enzyme activities. Starvation on the day of experiment did not result in any increased incorporation at 15:00h.

Therefore we will refer to the rates of RNA synthesis in rats killed at 09:00h as being low and those of the rats killed at 15:00h as being high.

Activities of extractable RNA polymerases in the liver nuclei in opposite feeding conditions

To ascertain whether increases in nuclear RNA synthesis were accompanied by parallel increases in the amount of extractable RNA polymerase activities, the latter were extracted as described in the Materials and Methods section, and separated by DEAE-Sephadex column chromatography, according to their differential elution by $(\text{NH}_4)_2\text{SO}_4$. The elution profiles of enzyme activities extracted from rats killed at 09:00h and at 15:00h are reported in Fig. 1. Two well-separated peaks of activity were found: the first (insensitive to α -amanitin; A enzyme) was eluted at 250 mM- $(\text{NH}_4)_2\text{SO}_4$. The second (sensitive to α -amanitin; B enzyme) was eluted at 350 mM- $(\text{NH}_4)_2\text{SO}_4$. Direct comparison of the elution profiles shows that the food intake is able to increase the total amount of form-B enzyme activities measured at 150 mM- $(\text{NH}_4)_2\text{SO}_4$ and with rat liver DNA being used as template.

The ability of form-B enzyme to transcribe deproteinized liver DNA over a large range of concentrations of $(\text{NH}_4)_2\text{SO}_4$ (Table 2) was investigated to establish whether food intake was able to modify the response of the enzyme to changes in ionic strength. The results are reported in Fig. 2. Form-B enzyme

Table 1. *Effect of food intake on the ability of rat liver nuclei to synthesize RNA in vitro in the presence or absence of α -amanitin*

Nuclei were isolated from the liver at 09:00h or at 15:00h from rats fed or denied food on the day of experiment. Portions of nuclear suspension, containing 90–100 μg of DNA, were assayed for their ability to incorporate [^3H]UTP as described in the Materials and Methods section. Radioactivity incorporated into acid-insoluble material was then estimated and expressed per mg of nuclear DNA. Data represent the average of six to eight determinations (three samples per determination) $\pm$ S.E.M. performed on different occasions.

Time of day (h)	Feeding condition	RNA polymerase activity (pmol of [^3H]UTP/mg of DNA)	
		Low-salt α -amanitin-insensitive reaction	High-salt α -amanitin-sensitive reaction
09:00	Starved since 17:00h of previous day	105 $\pm$ 10.2	248 $\pm$ 19.1
15:00	Fed since 09:00h	250 $\pm$ 16.8	416 $\pm$ 35.9
15:00	Starved on day of experiment	115 $\pm$ 11.3	236 $\pm$ 21.5

Fig. 1. Estimation of the amounts of extractable form-A and form-B RNA polymerase activity present in rat liver nuclei at 09:00h and at 15:00h

Liver (100g) from rats killed at either 09:00h or 15:00h were used to prepare form-A polymerase and form-B polymerase as described in the Materials and Methods section. The polymerase activities were separated on a DEAE-Sephadex column by using a linear gradient of 50–500mM-(NH₄)₂SO₄ (----). Portions (0.05 ml) from 2 ml fractions eluted from the column were assayed as described in the Materials and Methods section with rat liver DNA as template. Activity profiles obtained from rats killed at 09:00h (○) were expressed per 100mg of initial homogenate DNA and superimposed on profiles obtained from rats killed at 15:00h (●).

Table 2. Specific activity of form-B RNA polymerase extracted and purified from rat liver at different times of day, with deproteinized liver DNA as template at two different concentrations of (NH₄)₂SO₄

One unit of enzyme activity is defined as the amount of enzyme able to catalyse the incorporation of 1 pmol of [³H]UTP into the acid-insoluble product in 10 min, with rat liver DNA as template.

Time of day (h)	[(NH ₄) ₂ SO ₄] (mM)	...	Enzyme activity (units/mg of protein)	
			150	240
09:00			357	398
15:00			477	950

extracted from rats killed at 09:00h shows practically no sensitivity to the salt concentration, whereas the enzyme extracted from rats killed at 15:00h has a peak of activity between 200 and 260mM salt.

Characteristics of endogenous RNA polymerase activity in opposite feeding conditions

To obtain more information on the effect of food intake on RNA synthesis, chromatin-associated RNA polymerase was also investigated. Fig. 3 demonstrates

Fig. 2. Effect of various concentrations of (NH₄)₂SO₄ on form-B RNA polymerase activity extracted at 09:00h and 15:00h

DEAE-Sephadex column eluate (0.05 ml) was assayed in the presence of various concentrations of (NH₄)₂SO₄, as described in the Materials and Methods section, with deproteinized rat liver DNA as template. The activity pattern obtained from enzyme extracted at 09:00h (○) was expressed per 100 μg of initial homogenate DNA, and superimposed on the pattern obtained with enzyme extracted at 15:00h (●).

the salt dependence of endogenous activities of chromatins obtained from rats killed at 09:00h or at 15:00h. These experiments showed a minimal incorporation of label at low ionic strength for chromatins obtained from rats killed at both 09:00h and 15:00h and a maximum of activity at 240mM salt for chromatin extracted at 15:00h; chromatin extracted at 09:00h at this salt concentration reaches a plateau maintained throughout higher concentrations up to 400mM-(NH₄)₂SO₄. Addition of α -amanitin (1 μg/ml) to the incubation mixture results in a limited inhibition of activity at low salt concentrations and in a rather high inhibition (about 70%) over 240mM salt. This behaviour demonstrates that of the total endogenous activity only the form-B enzyme (α -amanitin-sensitive) is affected by salt concentrations, whereas form-A enzyme (α -amanitin-insensitive) is almost completely unaffected by salt concentrations in both chromatins studied.

The stimulation of endogenous form-B RNA polymerase activity by relatively high concentrations of (NH₄)₂SO₄ could be correlated with the removal of proteins from chromatin. Thus we have studied the release of proteins from chromatin by increasing concentrations of (NH₄)₂SO₄. Fig. 4 demonstrates that there is release of proteins as the ionic strength is increased and that chromatin extracted at 15:00h is in this respect more sensitive to ionic strength. Nevertheless, it must be pointed out that endogenous activity of chromatin extracted at 09:00h reaches a plateau at 240mM salt, although proteins are further released by higher concentrations of salt. The disparity between endogenous activity and the

Fig. 3. Salt dependence of the endogenous RNA polymerase activities of chromatin extracted at 09:00h or 15:00h

Chromatin (containing 50 μg of DNA) extracted from the rat liver nuclei at 09:00h (○) or at 15:00h (●) was assayed for the ability to incorporate [³H]UTP into RNA in various concentrations of (NH₄)₂SO₄. Reaction mixtures were incubated for 30min at 37°C and radioactivity incorporated into acid-insoluble material was then estimated per 100 μg of DNA. (a) Total activity; (b) α-amanitin-insensitive activity; (c) α-amanitin-sensitive activity.

release of proteins is even more evident in chromatin extracted at 15:00h.

Chromatin-associated RNA polymerase activity assayed at high ionic strength is regarded as representing the elongation step in RNA synthesis (Butterworth *et al.*, 1971). In our experimental conditions measurements of endogenous RNA synthesis performed at high ionic strength (240mM salt) did not show any significant difference in the presence or absence of the drug rifampicin AF/0-13 (Table 3). As this drug is able to inhibit the first internucleotide bond in *Escherichia coli* RNA (di Mauro *et al.*, 1969) and possibly in that of mammals (Butterworth *et al.*, 1971; Cox *et al.*, 1973) we did not find any re-initiation at high ionic strength.

Fig. 5 reports the kinetics of endogenous RNA synthesis for chromatins extracted at both 09:00h and 15:00h at 240mM salt. After 1h of incubation, the incorporation of radioactive precursor into RNA reached a plateau. This could be due to a cessation of

Fig. 4. Salt-dependent release of protein from chromatin extracted at 09:00h or 15:00h

Samples of liver chromatin extracted at 09:00h (○) or at 15:00h (●) (containing 60–80 μg of DNA) were treated with various concentrations of (NH₄)₂SO₄ as detailed in the Materials and Methods section. Proteins released were assayed by the method of Lowry *et al.* (1951). Values of total protein released are plotted as a function of the percentage of the total protein associated with the corresponding untreated chromatin.

Table 3. Effect of rifampicin AF/0-13 on chromatin-associated RNA polymerase activity

Chromatin-associated RNA polymerase activity was assayed as described in the Materials and Methods section by the use of chromatin extracted at two different times of day. Rifampicin AF/0-13 was added to the incubation medium (80 μg/ml) and the reaction followed for 30 min at 240mM-(NH₄)₂SO₄. Data are expressed as pmol of [³H]UTP incorporated/100 μg of DNA, and represent the average ± S.E.M. of six determinations (three assays per determination) performed on different occasions.

Time of day (h)	Rifampicin	...	Chromatin-associated RNA polymerase activity (pmol of [³ H]UTP/100 μg of DNA)	
			Absent	Present
09:00			12.8 ± 1.1	11.9 ± 0.8
15:00			22.1 ± 1.8	21.5 ± 1.9

RNA synthesis or to an equilibrium between RNA synthesis and degradation. A 50-fold dilution of [³H]UTP in the incubation mixture after 3h of incubation does not result in a loss of radioactivity from the acid-insoluble product, indicating that the degradation of the RNA product is essentially negligible. Nevertheless, more sensitive assays for RNAase* are needed before excluding the presence of RNAase activity in our chromatin preparations.

* Abbreviation: RNAase, ribonuclease.

Fig. 5. Comparison of kinetics of endogenous RNA polymerase activity of liver chromatin from rats killed at 09:00h or 15:00h

Reaction mixtures were incubated at 37°C up to 4h, and contained 50 µg of DNA as chromatin in a final concentration of 240 mM-(NH₄)₂SO₄. ○, Chromatin extracted at 09:00h; ●, chromatin extracted at 15:00h; ■, chromatin extracted at 15:00h from starved rats. After 3h, portions (10 µl) of 86 mM-unlabelled UTP were added (indicated by arrows) and the reaction was followed for another hour. (a) Total activity; (b) α-amanitin-insensitive activity; (c) α-amanitin-sensitive activity.

Effect of food intake on the efficiency of chromatin to act as template for exogenous form-B RNA polymerase

To evaluate whether the effect of food intake on liver RNA synthesis was also due to a modification of the chromatin template we have studied the efficiency of chromatins extracted at 09:00h or at 15:00h, to act as template for exogenous form-B RNA polymerase extracted from rats killed at 09:00h or 15:00h respectively, as a function of ionic strength. Chromatin was assayed in the absence of added enzyme as control.

Enzymes extracted from rats killed at both 09:00h and 15:00h are able to transcribe chromatin extracted from rats killed at 09:00h (Fig. 6a) and the added activity largely exceeds the endogenous one (Fig. 6b). Again a different sensitivity to salt

Fig. 6. Salt-dependence of form-B RNA polymerase activity extracted from liver nuclei at 09:00h or 15:00h with liver chromatin extracted at 09:00h as template

(a) Liver form-B RNA polymerase (20 units) extracted at 09:00h (○) or at 15:00h (●) and 50 µg of DNA as template were assayed at 37°C for 30 min in various concentrations of (NH₄)₂SO₄. Incubations to determine the endogenous activity of chromatin (□) contained samples of enzyme buffer. (b) RNA synthesis due to exogenous form-B enzyme extracted at either 09:00h (○) or 15:00h (●) was determined by subtracting the activity due to endogenous activity from total RNA synthesis.

concentrations is found for enzymes obtained at 09:00h and 15:00h. This difference greatly resembles that found by using deproteinized liver DNA as template, i.e. low salt-dependence of enzyme extracted at 09:00h, whereas enzyme extracted at 15:00h shows a peak of activity between 240 and 280 mM salt. This result is not in agreement with the findings of Butterworth *et al.* (1971) and Cox *et al.* (1973), who have indicated the range of concentrations between 150 and 180 mM salt as optimal for the initiation step of added form-B RNA polymerase.

A different behaviour of enzymes extracted from rats killed at 09:00h or at 15:00h has been found when they are assayed with chromatin extracted at 15:00h as template (Fig. 7). Enzyme obtained from rats killed at 09:00h is completely inhibited by 240 mM salt, but it is stimulated either by higher or lower concentrations. On the other hand, the enzyme obtained at 15:00h shows a peak of activity between 150 and 180 mM-(NH₄)₂SO₄ when assayed with the template extracted at the same time of day (Fig. 7).

Fig. 7. Salt dependence of form-B RNA polymerase activity extracted at 09:00h at 15:00h with liver chromatin extracted at 15:00h as template

(a) Liver form-B RNA polymerase (20 units) extracted at 09:00h (○) or 15:00h (●) and 50 μg of DNA as template were assayed at 37°C for 30 min in various concentrations of $(\text{NH}_4)_2\text{SO}_4$. Incubations to determine the endogenous activity of chromatin (■) contained samples of enzyme buffer. (b) RNA synthesis due to exogenous form-B enzyme extracted at either 09:00h (○) or 15:00h (●) was determined by subtracting activity due to endogenous activity from total RNA synthesis.

These different responses suggest that the concentration of $(\text{NH}_4)_2\text{SO}_4$ (between 150 and 180 mM) considered optimal for the initiation step (Butterworth *et al.*, 1971; Cox *et al.*, 1973) is really able to stimulate the initiation of new RNA chains to a maximum extent, maybe at very specific initiation points, only when the enzyme extracted at 15:00h transcribes chromatin obtained at the same time of day, and enzyme extracted at 09:00h possibly is able to initiate new RNA chains only at low ionic strength and maybe at non-specific initiation points.

To investigate further whether this different behaviour could be ascribed to an increased availability of binding sites for the enzyme on chromatin extracted from rats killed at 15:00h, we have evaluated the amount of liver DNA and chromatins extracted at 09:00h and 15:00h needed to saturate a fixed amount of enzyme obtained at 09:00h and

15:00h respectively (20 enzyme units). These experiments were performed at salt concentrations found to be optimal for initiation in our experimental conditions.

Results reported in Fig. 8(a) show that 20 units of enzyme extracted at 09:00h are readily saturated by liver DNA, whereas much larger concentrations of chromatin obtained at 15:00h, and even more obtained at 09:00h, are needed to saturate the same amount of enzyme extracted at 09:00h. Double-reciprocal plots (Fig. 8b) give very similar theoretical maximum velocities for all templates tested (Table 4), whereas the concentrations of DNA, and of chromatins from rats killed at 15:00h and 09:00h required to reach half the theoretical maximal velocity are very different. The apparent K_m values are 14.3 μM -deoxynucleotides for DNA, 53.4 μM for chromatin extracted at 09:00h and 32.2 μM for chromatin extracted at 15:00h.

A very similar behaviour was found for enzyme extracted at 15:00h (Figs. 8c and 8d) which is readily saturated by low concentrations of DNA, by larger concentrations of chromatin extracted at 15:00h, and even larger concentrations of chromatin extracted at 09:00h. Theoretical maximal velocity is lower when DNA is used as template (19 pmol of ^3H UTP incorporated/30 min) and higher (25 pmol of ^3H UTP/30 min) when either chromatin is transcribed (Table 4). The apparent K_m values reported in Table 3 are 13.5 μM -deoxynucleotides for DNA, 52.6 μM for chromatin extracted at 09:00h and 22.2 μM for chromatin extracted at 15:00h.

Discussion

Nuclei isolated from the liver of rats after 6h feeding following 16h starvation in the periodical re-feeding regimen of Potter *et al.* (1968) show an increased capacity to synthesize RNA. Intake of food enhances both low-salt α -amanitin-insensitive and high-salt α -amanitin-sensitive activities by 150 and 66% respectively.

The fluctuation of nucleoplasmic RNA synthesis measured in purified nuclei in the two extreme feeding conditions (i.e. 09:00h and 15:00h) is reflected in the increased activity of extractable form-B enzyme.

Oestradiol (Cox *et al.*, 1973) and other hormones (Mainwaring *et al.*, 1971; Smuckler & Tata, 1971; Yu & Fiegelson, 1971) are able to elevate form-B polymerase activity. However, no experiments have been reported on the sensitivity to ionic strength of form-B polymerase extracted from hormone-treated animals. The present data, which may indeed have a hormonal basis, are in this sense unique.

In an attempt to distinguish between food-intake effects at the enzyme level or on the functional state of the chromatin template, we have studied the

Fig. 8. Template dependence of liver form-B RNA polymerase extracted from rats killed at 09:00h or at 15:00h

(a) Liver form-B RNA polymerase extracted at 09:00h (20 units) was assayed in 90 mM $(\text{NH}_4)_2\text{SO}_4$, with various concentrations of deproteinized rat liver DNA (■), chromatin extracted at 09:00h (○) or 15:00h (●) as template. (c) Liver form-B RNA polymerase extracted at 15:00h (20 units) was assayed in 240 mM $(\text{NH}_4)_2\text{SO}_4$, with various concentrations of deproteinized rat liver DNA or chromatin extracted at 09:00h as template, or in 180 mM $(\text{NH}_4)_2\text{SO}_4$ with chromatin extracted at 15:00h as template. To determine RNA synthesis due only to exogenous enzyme radioactive isotope incorporation in assays containing no enzyme was subtracted for each concentration tested. Deproteinized or chromatin DNA concentrations are expressed as molarity of deoxynucleotides (average mol. wt. 309). (b) and (d) double-reciprocal plots of (a) and (c) respectively.

Table 4. Template-specificity of liver form-B RNA polymerase extracted at 09:00h or 15:00h from rat liver nuclei

Form-B RNA polymerase extracted at 09:00h or 15:00h was assayed with deproteinized rat liver DNA, or chromatins extracted at 09:00h or 15:00h as templates. For experimental details and salt concentrations see legend to Fig. 8 and the text. Data represent the average of four determinations performed on different occasions.

Enzyme extracted at (h)	Template (extracted at)	Apparent K_m (μM -deoxynucleotides)	Theoretical maximal velocity (pmol of UTP incorporated/30 min)
09:00	Deproteinized liver DNA (09:00h)	14.3	20.5
09:00	Chromatin (09:00h)	53.4	21.7
09:00	Chromatin (15:00h)	32.2	21.7
15:00	Deproteinized liver DNA (09:00h)	13.5	19.0
15:00	Chromatin (09:00h)	52.6	25.0
15:00	Chromatin (15:00h)	22.2	25.0

endogenous RNA polymerase activity bound to liver chromatin.

Food intake, after 16h starvation, is able to enhance the endogenous activity of form-A polymerase and form-B polymerase in chromatin by at least 50 and 70% respectively. This effect is not apparently due to gross differences in RNAase activity in chromatins, or to more extensive removal of proteins from chromatin extracted at 15:00h by $(\text{NH}_4)_2\text{SO}_4$. Since the re-initiation of RNA chains was negligible under conditions of endogenous RNA synthesis, the increase in endogenous activity seems to be due to a larger number of RNA polymerase molecules bound to chromatin. Nevertheless, with the present data one cannot exclude an increased elongation of RNA chains in chromatin, obtained from rats killed at 15:00h, although it seems reasonable to exclude it in view of the previous data of Cox *et al.* (1973) in oestrogen-treated chick oviduct.

In the absence of evidence to the contrary, it appears that this change may be ascribed, at least in part, to structural changes in the chromatin template.

To ascertain whether this behaviour could be ascribed to changes in the template and in the RNA polymerase enzymes we have transcribed the chromatins extracted at both 09:00h and 15:00h with the enzymes extracted at both 09:00h and 15:00h. The different optimum concentrations of $(\text{NH}_4)_2\text{SO}_4$ required by the same enzyme (extracted at 15:00h) to transcribe two different chromatins (240–260mm for chromatin extracted from rats killed at 09:00h, and 150–180mm for that extracted at 15:00h) also point to a modification of the chromatin template.

Possible modification of chromatin could be reflected in a different functional activity of the template and this was investigated by measuring the amount of different templates required to saturate a fixed amount of enzyme extracted at both 09:00h and 15:00h. The apparent K_m values demonstrate an increased availability of binding sites for both enzymes in the chromatin extracted at 15:00h.

The enzyme extracted from rats killed at 15:00h shows a greater affinity for its homologous template than does the one from rats killed at 09:00h, supporting the possibility of structural changes in the enzyme molecule induced by food intake. This is also consistent with the increased maximal velocity of the enzyme extracted from rats killed at 15:00h with respect to the enzyme extracted at 09:00h, as the theoretical maximal velocity is considered to be characteristic for a given enzyme.

We interpret the present results as indicating two different mechanisms involved in the modulation of RNA synthesis in the liver of rats on controlled feeding schedules. One mechanism involves the apparent structural modification of the enzyme. This becomes evident because of the different sensitivity to

ionic strength and the different affinity for the chromatin template. This interpretation is consistent with the data of Stein *et al.* (1973), who report the presence of a cytoplasmic protein which binds the mammalian RNA polymerase thereby increasing the enzyme activity and shifting the salt concentration optimum of the reaction (Stein & Hausen, 1970).

The second mechanism involves the availability of DNA chromatin template. We interpret this as a modification (or reorganization) of the protein–DNA complex to allow the transcription of new or additional RNA products. Nevertheless, at the moment it is not clear whether this is obtained by new promoter sites for modified enzyme binding, or more efficient transcription of already available cistrons, by structural changes of histone and/or non-histone proteins, which may have a regulatory function of gene expression (Widnell & Tata, 1964, 1966; Teng & Hamilton, 1968; Elgin *et al.*, 1971).

We are grateful to Professor G. Moruzzi for his interest in this research, and for providing all the facilities and the financial support, to Dr. R. F. Cox of Searle Research Laboratories for helping B.T. in the enzyme preparation, and to Mr. G. Negroni for his excellent technical assistance.

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