Mechanism of enhanced RNA synthesis in acute-phase rat liver and its relationship to chromatin structure

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Nuclei isolated from the liver of rats undergoing an acute inflammatory reaction induced by turpentine treatment show increased RNA synthesis. This increase is essentially determined by a faster polyribonucleotide-elongation rate while the number of transcribing polymerase molecules is unchanged. The sensitivity of chromatin to micrococcal-nuclease digestion and the composition of chromosomal proteins are not affected by the acute-phase process. Therefore the increased RNA synthesis by liver nuclei from acutely inflamed rats does not seem to correlate with major changes in chromatin structure.

The co-ordinate increase in concentration of some plasma proteins, known as 'acute-phase reactants' occurring during the inflammatory response (Koj, 1970) depends on a specific activation of their synthesis in the liver (Kushner & Feldmann, 1978; Courtoy *et al.*, 1981). This activation is in turn correlated with an increase in RNA synthesis, accompanied by re-programming of RNA transcription and eventually by an increase in the relative amounts of the specific mRNA molecules for 'acute-phase reactants'; transcriptional regulation seems to be the single most important mechanism in the onset of 'acute-phase reactants' production (Princen *et al.*, 1981; Ricca *et al.*, 1981).

In the present paper we investigate the molecular mechanisms which can lead to modifications of transcription in rat liver during the acute inflammatory reaction. RNA synthesis by isolated nuclei *in vitro* can be influenced by the composition of the medium (ionic strength, type of ions, presence of effectors, etc.), but, under defined experimental conditions, it depends essentially on two factors and their possible interactions: (i) activity of transcribing RNA polymerases; (ii) capacity of the chromatin to serve as template for the polymerases.

Possible changes in polymerases activity in rat liver during the inflammatory reaction induced by

subcutaneous turpentine treatment can be due to changes in elongation rate and/or difference in the number of transcribing polymerase molecules. In the present work we calculated the number of polymerase molecules engaged in each transcription unit and the rate of elongation of the nascent polynucleotide chains; calculation was based on UMP/uridine analysis of the alkali-digested nascent RNA, synthesized in the presence of labelled UTP by isolated liver nuclei from turpentine-treated rats. By using this technique we were able to define the basis of increased polymerase activity in ischaemic (Schiaffonati *et al.*, 1982), post-ischaemic (Cairo *et al.*, 1981) and regenerating liver nuclei (Piccoletti *et al.*, 1982). More recently a quantitative morphometric analysis of the number of transcriptional units and their associated RNP fibrils in liver nuclei during the inflammatory reaction has been done by means of electron microscopy (Harper *et al.*, 1983).

The regulation of gene expression seems to be directly related to chromatin structure. Nuclease-digestion experiments have demonstrated that the conformation of active nucleosomes is different from that of bulk nucleosomes (Weisbrod, 1982). Moreover, many studies have suggested that the active structure is due to altered or modified histones and/or the presence of non-histone chromatin proteins in the nucleosome (Allfrey, 1977). In order to investigate whether the altered transcriptional capacity in the liver nuclei during the acute-

Abbreviations used: SDS, sodium dodecyl sulphate; RNP, ribonucleoprotein.

phase response is correlated with changes in chromatin structure, we determined the susceptibility to nuclease digestion and the composition in histones and some non-histone proteins of isolated chromatin. The results of the present investigation suggest that an increase in the elongation rate of the nascent polynucleotide chains accounts for the increased RNA synthesis by nuclei isolated from turpentine-treated rat liver. This occurs without major changes in chromatin conformation, although minor, but specific, modifications to particular regions of the chromatin would not be detectable by the methods used in this work.

Materials and methods

Animals

Male albino rats (Wistar strain) weighing approx. 200g were maintained with alternating 12h cycles of light and darkness, and fed on a balanced diet *ad libitum*. Acute inflammation was induced by a single subcutaneous injection of turpentine oil (0.5ml/100g body wt.) into the back. Rats were killed 16h after the injection.

Chemicals

[5-³H]UTP (ammonium salt; sp. radioactivity 10–30Ci/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Unlabelled nucleotides, actinomycin D and α -amanitin were from Boehringer G.m.b.H., Mannheim, West Germany. Phenylmethanesulphonyl fluoride and micrococcal endonuclease (EC 3.1.31.1; grade VI) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; 1 unit is the amount of enzyme that produced 1 μ mol of acid-soluble polynucleotides from DNA/min at pH 8.8 and 37°C. Polyethyleneimine–cellulose plates were from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). All other reagents were of the highest purity available.

Isolation of nuclei

The livers were quickly removed and washed in cold homogenization medium appropriate for each procedure. All subsequent operations were performed in the cold. For UMP/uridine analysis and preparation of chromatin, nuclei were isolated as described by Widnell & Tata (1964). For determination of sensitivity to micrococcal nuclease, nuclei were prepared by a modification of the procedure described by Jackson *et al.* (1979). Briefly, livers were homogenized in 3 vol. of buffer A [50 mM-Tris/HCl (pH 7.4)/3 mM-KCl/5 mM-MgCl₂/0.2 mM-CaCl₂/0.1% (v/v) Triton X-100/1 mM-phenylmethanesulphonyl fluoride] containing 0.32 M-sucrose with an Ultra-Turrax homogenizer (type 18-10, Janke und Kunkel K.G. Staufen

i. Breisgau, West Germany) at medium speed for 15 s. The homogenate was filtered through cheesecloth, diluted to 0.25 M-sucrose with buffer A and centrifuged at 900g (r_{av} 15 cm) for 5 min to obtain a crude nuclear pellet. The pellet was resuspended in buffered 2.4 M-sucrose (without the Triton X-100) and centrifuged at 36800g (r_{av} 8.1 cm) for 1 h. The purified nuclear pellet was then washed twice in 1 vol. of nuclei-wash buffer [10 mM-Tris/HCl (pH 7.5)/250 mM-NaCl/50 mM-NaHSO₃/1 mM-phenylmethanesulphonyl fluoride].

Preparation of chromatin

The nuclear pellet obtained as described above was resuspended in 50 mM-Tris/HCl (pH 8) and chromatin was prepared by stepwise decrease in ionic strength as described by Pierce & Fausto (1978). When chromatin was prepared for protein extraction, 0.5 mM-phenylmethanesulphonyl fluoride was included in all buffers.

Protein extraction and electrophoretic analysis

Chromosomal proteins were extracted for 2 h at 4°C with 0.2 M-H₂SO₄/0.5 mM-phenylmethanesulphonyl fluoride/2 mM-sodium butyrate, dialysed against 1 mM-HCl/0.1 mM-sodium butyrate and freeze-dried. Electrophoretic analyses were done on polyacrylamide gels (1.5 mm $\times$ 30 cm) in 0.8 M-acetic acid/4 mM-urea essentially by the procedure of Oliver & Chalkley (1972). The stacking gel was as described by Spiker (1980), the slots were filled with 30 μ l of 1 M-cysteamine [dissolved in the stacking solvent plus 15% (w/v) sucrose and 5% (v/v) β -mercaptoethanol], and proteins were accumulated at the interface by running at 50 V for 2 h. Electrophoresis was performed for about 40 h at 140 V. The gels were fixed and stained in methanol/water/acetic acid (5:4:1, by vol.) plus 1.5% (w/v) Coomassie Blue R250 for 4 h at room temperature and destained in ethanol/water/acetic acid (25:65:8, by vol.). Gels to be used in the second dimension were soaked in destaining solution containing 5% (v/v) glycerol and kept sealed at 4°C.

For second-dimension electrophoresis, bands were excised from the acetic acid/urea gels, incubated for 30 min in gel solvent containing 1% (w/v) SDS, and placed on top of a 20 cm-long 15% (w/v)-polyacrylamide slab gel containing 0.1% SDS. The electrophoresis was performed by the method of Laemmli (1970) as modified by Thomas & Kornberg (1975) for 14 h at 110 V, and stopped when the Coomassie Blue of the first-dimension bands reached the bottom of the gel. Gels were fixed and stained with silver staining by the following method. After electrophoresis the gels were fixed for at least 3 h in methanol/water/acetic acid (5:4:1, by vol.) and overnight in methanol/water (1:1, v/v) containing 1% (v/v) formaldehyde. The

gels were then equilibrated in distilled water, which was changed many times over a period of at least 8 h, and soaked for 25 min with gentle shaking in a solution prepared by mixing 280 ml of 0.08% (w/v) NaOH/0.6% (v/v) NH_3 with 1.5 g of AgNO_3 dissolved in 20 ml of water. After a quick rinse with water, the bands were developed with 0.005% citric acid/0.05% formaldehyde. The staining was stopped by washing with water and soaking in methanol/water/acetic acid (5:4:1, by vol.) for 30 min. Finally, the gels were equilibrated in ethanol/water/acetic acid (25:65:8, by vol.) containing 5% glycerol. This last procedure allows one to clean the gels with a paper tissue wetted in the same solvent.

UMP/uridine analysis

RNA synthesis was performed in a standard incubation mixture of 0.25 ml containing 83 mM-Tris/HCl (pH 7.8)/1.66 mM- MgCl_2 /23 mM- β -mercaptoethanol/66 mM- $(\text{NH}_4)_2\text{SO}_4$ /0.4 mM-ATP/0.4 mM-GTP/0.4 mM-CTP/0.05 mM- $[\text{}^3\text{H}]\text{UTP}$ (sp. radioactivity 1.2 Ci/mmol). When appropriate, α -amanitin was added at a concentration of 2 $\mu\text{g}/\text{ml}$ to measure RNA synthesis by polymerase I; RNA synthesis by polymerase II was calculated by subtracting α -amanitin-insensitive RNA synthesis (polymerase I) from total RNA synthesis, measured in the absence of the inhibitor. Precipitation, washing, hydrolysis and chromatography of RNA synthesized *in vitro* were performed exactly as described by Coupar *et al.* (1978). Radioactivity on the section of the plates containing UMP and uridine residues was determined as described by Cairo *et al.* (1981). Average recovery of nuclear UTP was 82%.

Elongation rates and number of transcribing RNA chains being synthesized per diploid genome were calculated as described (Coupar *et al.*, 1978).

DNA was determined by the diphenylamine procedure (Burton, 1956), on separate samples of the preparation of nuclei.

Micrococcal-nuclease digestion

Digestion of nuclei was performed as described by Re *et al.* (1983). Nuclei were suspended to the concentration of $15A_{260}$ units/ml in 15 mM-Tris (pH 8.0 adjusted with solid cacodylic acid)/15 mM-NaCl/60 mM-KCl/10 mM- MgCl_2 /1 mM- CaCl_2 /1 mM-phenylmethanesulphonyl fluoride and treated with micrococcal nuclease (0.050 unit of activity/ A_{260} unit of nuclei) for different times at 30°C.

Percentage solubility was measured as previously described (Re *et al.*, 1983).

Statistical analysis

Significance of differences was assessed by Student's *t* test.

Results

Polynucleotide-elongation rates and number of transcribing polymerase molecules

These can be calculated from the amounts of $[\text{}^3\text{H}]\text{UMP}$ and $[\text{}^3\text{H}]\text{uridine}$, derived from internal nucleotides and 3'-terminal nucleosides respectively, of polynucleotide chains synthesized in the presence of $[\text{}^3\text{H}]\text{UTP}$ during 1 min incubation. Newly synthesized RNA is subjected to alkaline digestion, and UMP and uridine are then separated by t.l.c. (Coupar *et al.*, 1978). Of the possible events that could lead to incorrect results, initiation does not occur appreciably in isolated nuclei, which can only elongate RNA chains already initiated *in vivo* (Grummt & Lindigkeit, 1973; McReynolds & Penman, 1974). The intracellular concentration of UTP, less than 1 μM , as estimated for liver nuclei (Coupar *et al.*, 1978), is negligible with respect to the final concentration of UTP in the medium. The possibility of ribonuclease activity during RNA synthesis was tested by adding actinomycin D and α -amanitin to inhibit RNA synthesis after the 1 min incubation and performing the incubation for 1 min more (Coupar & Chesterton, 1977); no significant ribonuclease activity was ever detected in either normal or turpentine-treated rat liver nuclei. A certain amount of degradation of UMP to uridine during the alkaline hydrolysis and processing of nuclei, not exceeding 0.5% of the total, was taken account of in the calculation.

When these requirements are met, the method used in the present investigation allows a faithful estimation of both parameters of RNA synthesis.

The experimental values of $[\text{}^3\text{H}]\text{uridine}$ and $[\text{}^3\text{H}]\text{UMP}$ residues obtained after hydrolysis and separation are reported in Table 1(a).

The greater amount of radioactivity incorporated into nascent polynucleotide chains is found in internal UMP residues, which account for most of the RNA synthesis. Total RNA synthesis, as well as the fraction dependent on the activity of polymerase I, which under the present experimental conditions represents the larger amount of the total, seem to be significantly increased in acute-phase liver; no changes are found in the labelling of uridine residues. Calculations from these values give the true results of the experiments, i.e. the number of polymerases transcribing and their rate of elongation, which are reported in Table 1(b). These results show that during the acute-phase reaction there is no change in the number of transcribing polymerases; on the contrary, the elongation rate is significantly increased for both polymerase I- and polymerase II-dependent RNA synthesis. The percentage increase in the rate of

Table 1. Effect of turpentine treatment on radioactivity present in internal UMP and 3'-terminal uridine of the RNA synthesized by isolated liver nuclei (a) and on transcribing polymerases and polyribonucleotide elongation rate in isolated liver nuclei (b)

Separation by t.l.c. and determination of radioactivity of internal UMP and 3'-terminal uridine of RNA synthesized by form-I and form-II polymerases in isolated liver nuclei were performed as described in the Materials and methods section. The number of transcribing polymerases and polyribonucleotide-elongation rate were calculated from [^3H]UMP and [^3H]uridine residues as described in the Materials and methods section. Values represent means $\pm$ S.E.M. of four experiments: * $P < 0.05$; ** $P < 0.01$.

(a)	Rats	[^3H]UMP (c.p.m./ μg of DNA)			[^3H]Uridine (c.p.m./ μg of DNA)		
		Total	Form I	Form II	Total	Form I	Form II
	Control	115 $\pm$ 12.2	72 $\pm$ 7.8	43 $\pm$ 3.8	5.5 $\pm$ 0.9	3.6 $\pm$ 0.74	1.9 $\pm$ 0.76
	Turpentine-treated	182.8 $\pm$ 19**	130.3 $\pm$ 15.2**	50.2 $\pm$ 4.8	5.0 $\pm$ 0.7	3.4 $\pm$ 0.6	1.6 $\pm$ 0.13
(b)	Rats	10 $^{-3}$ $\times$ No. of transcribing RNA polymerase molecules (per diploid genome)			Polyribonucleotide-elongation rate (no. of nucleotides/s)		
		Total	Form I	Form II	Total	Form I	Form II
	Control	63.2 $\pm$ 5.4	48.4 $\pm$ 3.3	14.8 $\pm$ 2.4	0.35 $\pm$ 0.04	0.33 $\pm$ 0.04	0.39 $\pm$ 0.02
	Turpentine-treated	57.5 $\pm$ 4.7	45.2 $\pm$ 3.7	12.3 $\pm$ 2.1	0.61 $\pm$ 0.03**	0.64 $\pm$ 0.06**	0.52 $\pm$ 0.05*

elongation by polymerase I seems to be higher than that by polymerase II.

In the interpretation of these results, the following points should be considered: (i) at the concentration of 2 $\mu\text{g}/\text{ml}$, α -amanitin does not inhibit polymerase III, which is therefore calculated together with polymerase I; the activity of form III, however, represents only a small fraction of the total (Lampert & Feigelson, 1974); (ii) the balance between the respective fractions of activity ascribable either to polymerase I or polymerase II is largely dependent on conditions superimposed *in vitro*. This seems to be the main reason why, under circumstances of undisputed increases in RNA synthesis, the contributions of form-I and form-II polymerases to the increase are sometimes controversial.

Apart from these minor points, it seems clear that the increase in the rate of elongation, rather than in the number of enzyme molecules engaged in transcription, is responsible for the stimulation of RNA synthesis during the acute-phase reaction. This is at variance with the events occurring in liver regeneration (Piccoletti *et al.*, 1982) or in post-ischaemic repair (Cairo *et al.*, 1981), where an increase in the number of transcribing enzyme molecules is mainly responsible for the increased RNA synthesis by isolated nuclei.

More recent observations by electron microscopy suggest that in acute-phase livers the numbers of sites of RNA synthesis along the DNA strand are either unchanged or possibly decreased (Harper *et al.*, 1983). The enhancement of the elongation rate demonstrated in our experiments is therefore the most likely explanation of the increased RNA synthesis occurring during the acute-phase reaction.

Nuclease sensitivity

Changes in chromatin conformation are among the numerous factors controlling the extent and the selectivity of transcription. Enhanced accessibility of DNA to endonuclease attack is considered to reflect these structural changes. This occurs in hypertrophic heart from spontaneously hypertensive rats (Limas, 1982) or in thyroid after stimulation by thyrotropin (Abe *et al.*, 1982), where enhanced RNA synthesis is accompanied by increased susceptibility of isolated nuclei to digestion by deoxyribonuclease I. Liver nuclei from animals treated with angiotensin also showed an increased solubility of chromatin by micrococcal-nuclease treatment (Re *et al.*, 1983). Nuclei isolated from turpentine-treated animals and controls were re-suspended in buffer with or without micrococcal nuclease and incubated for the indicated time periods (Fig. 1). Nuclei obtained from turpentine-treated rats do not show increased solubility compared with controls in either the presence or the absence of the enzyme. Micrococcal nuclease was chosen because it seems to give the best results, not easily influenced by changes in the isolation conditions for the nuclei (Prentice & Gurley, 1983). But essentially the same results, i.e. no changes in the sensitivity of acute-phase rat liver nuclei, were obtained by digestion with deoxyribonuclease I (results not shown).

Chromosomal protein composition

'Active' chromatin has been reported to be correlated with altered or modified histones and/or the presence of non-histone chromosomal proteins in the nucleosome. Moreover, post-synthetic histone modification by phosphorylation, methyla-

Fig. 1. Effect of turpentine treatment on solubility of rat liver nuclear DNA by micrococcal-nuclease digestion. Nuclei ($15 A_{260}$ units/ml) from control (○) and turpentine-treated (●) rat livers were incubated at 30°C with (—) or without (----) micrococcal nuclease for the indicated times. Digestion conditions and determination of percentage of solubility were as described in the Materials and methods section. Values represent means of three experiments.

tion and acetylation (Allfrey, 1977, 1980) and different amounts of histone $H1^0$ (D'Anna *et al.*, 1982), non-histone proteins HMG 1, HMG 2 and A 24 (Goldknopf & Bush, 1978; Goodwin & Mathew, 1982) can lead to severe alterations in chromatin structural organization.

In particular, increased histone-H1 phosphorylation and markedly decreased amounts of protein A 24 were found in actively transcribing cells such as regenerating rat liver hepatocytes (Laks *et al.*, 1981; Ballal *et al.*, 1975). We therefore analysed the electrophoretic pattern of chromosomal proteins extracted from control and acutely inflamed rat liver. We found that proteins extracted from control and turpentine-treated rat liver present the same pattern by one- and two-dimensional electrophoretic analysis. A typical electrophoretic result is reported in Fig. 2. It is noteworthy that the considerable amounts of protein A 24, relative to HMG 1 and HMG 2, are characteristic of rat liver chromatin.

Moreover, no difference was found in these non-histone proteins, or in the amount of histone $H1^0$, between control and turpentine-treated animals. In addition, the lack of appearance of bands between those of $H1^0$ and H3, and of H2A and H4,

Fig. 2. Electrophoretic analysis in acetic acid/urea gel of acid-soluble proteins extracted from control or turpentine-treated rat liver chromatin

First-dimension electrophoresis was performed in 15%-polyacrylamide/acetic acid/urea gel (1D/AcOH/urea). The bracketed region of the gel was then applied on a 15%-polyacrylamide gel containing 0.1% SDS and subjected to second-dimension electrophoresis (2D/SDS). Electrophoresis and staining of protein bands were performed as described in the Materials and methods section.

shows that there is no acetylation of histones H3 and H4, respectively, in both control and acutely inflamed rat liver.

However, other variations in the distribution of some chromosomal proteins of higher molecular weight not influencing the basic structure of chromatin, but affecting the extent of RNA synthesis, could be present in turpentine-treated animals.

Discussion

Analyses by nuclease-sensitivity and chromosomal protein composition are internally consistent and exclude major changes in the state of liver chromatin during the acute-phase reaction. This is at variance with physiological conditions, where increases in RNA synthesis, preceding cell multiplication (Piccoletti *et al.*, 1982) or cell hypertrophy (Limas, 1982; Abe *et al.*, 1982) are associated with changes in the physical properties of the chromatin template. It seems possible to us to conclude that the acutely inflamed rat liver represents a cellular system in which there is activation of RNA transcription without a concurrent increase in nuclease sensitivity. This may be explained by the enzymic mechanism which leads to the increased incorporation of precursor nucleotides into RNA, i.e. faster elongation rate without alterations in RNA polymerase accessibility. This could be accomplished leaving unaltered the regions particularly sensitive to nuclease attack, which map at the 5' end of the gene, where the start site of transcription is localized. However, under the conditions of the present investigation, other transcriptionally relevant changes in chromatin structure could have escaped detection.

The increase in RNA synthesis by nuclei from turpentine-treated rats causes an increased overall production of rRNA, which later accumulates in the cytoplasm in the form of membrane-bound polyribosomes. The events can be different as regards mRNA; increased synthesis of precursors of mRNA is less pronounced, and is likely to cause a new balance in the relative amounts of mRNA molecules rather than an absolute increase in all the mRNA species. From this point of view it is relevant that the accumulation of mRNA species coding for the acute-phase reactants is accompanied by a sizeable decrease in the amount of mRNA coding for albumin (Princen *et al.*, 1981; Bardella *et al.*, 1983). This change in the population of the liver mRNA could be reached either by increase in number of RNA polymerase molecules transcribing mRNA for acute-phase reactants, with concurrent decrease in number of polymerase molecules transcribing albumin mRNA, or by a preferential stimulation of elongation of mRNA

for acute-phase reactants, or by a combination of the two processes.

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