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Histone Phosphokinase Activity in Nuclear and Cytoplasmic Cell Fractions from Normal and Regenerating Rat Livers

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1. Liver cell fractions were prepared by non-aqueous procedures and nuclei were also obtained in a hyperosmotic sucrose medium. Histone phosphokinase activity, assayed with histone F1 as substrate, was present in the soluble fraction of the cytoplasm and also bound on to the chromatin fraction of the nucleus. 2. The activity of the enzyme increased sixfold in nuclei from regenerating livers 22h after partial hepatectomy. 3. The enzyme bound in the nucleus was only marginally activated by 1μM-3′:5′-cyclic AMP which stimulated the cytoplasmic soluble enzyme fourfold. 4. Nuclei prepared by the non-aqueous technique were also able to phosphorylate histones F2a and F3 and showed histone phosphatase activity with histone F1 phosphate as substrate.

Histone F1 undergoes changes in its phosphorylation state *in vivo* that depend on the physiological or the experimental condition of the organism (Stevely & Stocken, 1968). Such changes could contribute to the modified histone–DNA interactions necessary for information transfer.

A histone F1 phosphokinase has been observed in many tissues in the cytosol and is thought to be specific for histones. Phosphorylation of histone F1 by this enzyme has been studied *in vitro* in several laboratories (Langan & Smith, 1967; Langan, 1969*a*,*b*; Pawse, Ord & Stocken, 1971).

Although soluble enzymes are usually found also in the nucleus (Siebert & Humphrey, 1965; Siebert, 1968), a study was undertaken to localize more precisely the histone phosphokinase in subcellular fractions from rat liver. We have found that, besides the above-mentioned soluble phosphokinase, rat liver nuclei contain a histone phosphokinase that is firmly bound to insoluble nuclear constituents, probably to chromatin.

MATERIALS AND METHODS

Nuclei and other cell fractions. Female Sprague–Dawley rats, 180–200g weight, were obtained from Ivanovas Animal Farm, Kisslegg-Allgäu, W. Germany. After 16h starvation, the animals were partially hepatectomized (Higgins & Anderson, 1931) and the excised liver lobes were quick frozen in liquid N_2. Regenerating liver was obtained from rats 20–22h after operation and was treated similarly. Nuclei and other cell fractions were prepared by a non-aqueous procedure and analysed by a modification of the Behrens technique (Siebert, 1967). The composition of the cell fractions obtained by the non-aqueous isolation procedure is shown in Table 1.

For the preparation of sucrose nuclei, male Wistar rats, 180–200g weight, of the laboratory strain (Oxford), were subjected to partial hepatectomy or sham operation without starvation. Nuclei were prepared in high-density sucrose as described by Chauveau, Moulé & Rouiller (1956). High-speed supernatants were obtained from 22% (w/v) homogenates of livers from identically treated animals in 0.25M-sucrose by centrifuging for 60min at 20000*g* and then at 105000*g*.

Histone kinase assay. For enzyme assays with material obtained by the non-aqueous procedure, 30–40mg of parenchymal powder, solvent-treated parenchymal powder or cytoplasm, or 25–30mg of nuclei were suspended at 0°C in 1ml of 0.14M-NaCl–33mM-tris–HCl buffer (pH7.4)–3mM-$MgCl_2$ for 20–30min. Extracts were prepared by centrifugation at 5000*g* for 30–60min and used as enzyme sources. Nuclei were also used as a suspension. Nuclear residues were prepared by resuspending the nuclear sediment after similar centrifugation, in the original volume of the above-mentioned saline solution. The methods used for extraction and subfractionation of nuclei obtained in 2.2M-sucrose are described in Table 3.

The assay conditions closely follow methods described for histone F1 by Pawse *et al.* (1971) and for histone F2a and F3 by Mr P. H. Campbell (personal communication). In a total volume of 1.5ml were incubated, for 20min at 25°C, 1mg of histone, 50μmol of tris–HCl buffer, pH7.4, 5μmol of $MgCl_2$, 0.5μmol of [γ-^{32}P]ATP, 1.5nmol of 3′:5′-cyclic AMP, 0.5μmol of reduced glutathione and 0.5–2mg of enzyme protein. The reaction was stopped with trichloroacetic acid at 5% (w/v) final concentration for experiments with histone F1 and at 25% (w/v) final concentration with histones F2a and F3. The 5% trichloroacetic acid supernatant was combined with a second trichloroacetic acid extract of the sediment and brought to 25% final concentration to precipitate histone

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Table 1. *Composition of liver-cell fractions obtained by the non-aqueous procedure*

A detailed account of the fractionation is given by Siebert (1967). Nucleic acid contents are given as % dry weight.

	Control			Regenerating		
Cell fraction	DNA %	RNA %	RNA/ DNA	DNA %	RNA %	RNA/ DNA
Parenchymal powder	0.93	2.23	2.4	0.88	2.99	3.4
Solvent-treated parenchymal powder	0.84	2.70	3.2	0.95	3.93	4.1
Cytoplasm	0.34	3.49	8.6	0.30	4.12	13.7
Nuclei	11.5	2.24	0.19	10.6	3.55	0.35

F1. For histones F2a and F3, the trichloroacetic acid precipitate was extracted three times with 0.1 M-H_2SO_4, and the combined extracts were adjusted to 25% trichloroacetic acid for histone precipitation. Reprecipitation of histone F1 was done three times and of histones F2a and F3 twice. ^{32}P radioactivity was determined on Čerenkov radiation in a Beckman Liquid-Scintillation Counter with an efficiency of 30%. (Sufficient counts were recorded to give an accuracy of not less than ±3%.) Blank incorporation never exceeded 2% of the assay values. The results are expressed as nmol of ^{32}P incorporated in 20 min.

Histone phosphatase in cell fractions isolated by the non-aqueous procedure. In a total volume of 1.3 ml were incubated, at 25°C, 1 mg of ^{32}P-labelled histone F1, 100 μmol of tris–HCl buffer, pH 7.4, 10 μmol of $MgCl_2$, 0.5 μmol of reduced glutathione, 200 μmol of NaCl, and 0.5–1.5 mg of enzyme (Meisler & Langan, 1969). The reaction was stopped by addition of 0.5 ml of 100% (w/v) trichloroacetic acid after 5 or 20 min, and the sediments were washed with 0.7 ml of 25% trichloroacetic acid. P_i in the combined supernatants was converted into phosphomolybdate, and extracted into 2-methylpropan-1-ol (Berenblum & Chain, 1938) for counting of radioactivity.

Histone substrates. Crude nuclei were prepared from 300 g of fresh sheep thymus in 0.25M-sucrose–5 mM-tris–HCl (buffer pH 7.2) 5 mM-$MgCl_2$–washed twice with 10 mM-tris–HCl(buffer pH 7.2)–5 mM-$MgCl_2$and once with 0.5 mM-H_2SO_4. By using Johns's (1964) method 1, histone F1 was extracted with 150 ml of 5% (w/v) $HClO_4$, re-extracted with 50 ml of 5% $HClO_4$, and the combined extracts were precipitated at a final concentration of 25% (w/v) trichloroacetic acid. The sediment was dissolved in 120 ml of 5% $HClO_4$, reprecipitated as above, taken up in 35 ml of water and dialysed against two changes of 70 vol. of water. The final solution was clarified at 40 000 *g* for 20 min and stored at −20°C. The yield was 560 mg.

By adapting Johns's (1964) method 2, the nuclear residues that remained after $HClO_4$ extraction was washed with ethanol, extracted with 150 ml of ethanol–1.25M-HCl (4:1, w/v) and the extract dialysed against 500 ml of ethanol. Histone F3 was sedimented and after centrifugation the supernatant was rapidly mixed with 5 vol. of acetone at −20°C to precipitate histone F2a. Each sediment was dissolved in 15 ml of water and dialysed overnight against 300 vol. of water. Histone F3 was clarified by high-speed centrifugation. This method yielded 220 mg of histone F3 and 200 mg of histone F2a. The histones were stored at −20°C.

Amino acid analysis and gel electrophoresis were used as criteria to establish the purity of the preparations. Histone F1 was judged to be not less than 90% pure; histone F2a not less than 80% pure and histone F3 not less than 75% pure.

Protein was determined as described by Lowry, Rosebrough, Farr & Randall (1951) and DNA by the method of Burton (1956).

Preparation of ^{32}P-labelled histone F1. ^{32}P-labelled histone F1 was conveniently re-isolated from assays for phosphokinase activity where at least 0.5 nmol of P/mg of histone had been incorporated.

γ-^{32}P-labelled ATP was prepared as described by Glynn & Chappell (1964); all other chemicals were commercial products of AnalaR purity.

RESULTS

Histone phosphokinase activity is retained in cell fractions isolated by the non-aqueous procedure (Table 2). Extraction of equivalent amounts of parenchymal powder and fresh liver gave about twice as much activity in the supernatant from the fresh liver but this might have been due in part to the different strains of rats used in the two preparations. The enzyme activity in the parenchymal powder on a dry weight basis is slightly decreased by solvent treatment and a slightly greater inactivation may be occurring in preparations from regenerating livers. Enzyme activity is found both in the cytoplasm and bound on to nuclei; the activity/dry weight is of the same order of magnitude for both cell fractions.

The presence of enzyme bound on to nuclei prepared by the non-aqueous technique suggests that the enzyme should be detected after isolation through hyperosmotic sucrose. The minced liver was homogenized directly in 2.2M-sucrose; there was no preliminary exposure of the nuclei to iso-osmolar sucrose. Considerable histone phosphokinase activity was found in liver nuclei isolated in this way (Table 3) and the activity of the enzyme was sixfold higher 22 h after regeneration. Between 2 and 8% of the ^{32}P was transferred on to endogenous histone F1 of nuclear fractions which con-

Table 2. *Histone phosphokinase activity in non-aqueous cell fractions from rat liver*

The fractions were obtained as described by Siebert (1967).

Cell fraction	Control Sp. activity (nmol of ^{32}P incorporated) (per mg dry wt.)	(per mg of protein)	(per mg of DNA)	Regenerating Sp. activity (nmol of ^{32}P incorporated) (per mg dry wt.)	(per mg of protein)	(per mg of DNA)
Parenchymal powder	0.35	0.92	38	0.40	1.63	45
Solvent-treated parenchymal powder	0.32	0.98	38	0.32	1.20	34
Cytoplasm	0.23	0.97	68	0.24	0.96	79
Nuclear suspension	0.42	—	3.7	0.22	—	2.1
Nuclear extract	0.03	0.36	—	*	*	*
Nuclear residue	0.41	—	3.6	0.17	—	1.6

* Too low for reliable assay.

Table 3. *Histone phosphokinase activity in subfractions of rat liver nuclei isolated in high-density sucrose*

For these experiments, nuclei derived from one normal or two partially hepatectomized rats were mixed with 2.0ml of 0.25M-sucrose–0.14M-NaCl–33mM-tris–HCl buffer (pH 7.4)–3.3mM-$MgCl_2$ by thorough homogenization in a Dounce homogenizer. The gel-like material obtained was designated nuclear suspension. In some instances the nuclear suspension was centrifuged so as to remove insoluble material, nuclear membranes etc. The nuclear suspension was diluted with enough (approx. 6 vol.) redistilled water to render nucleoprotein insoluble; this fraction was separated by centrifugation and designated as chromatin and the supernatant, saline extract. The chromatin fraction was resuspended in 0.14M-NaCl–33mM-tris–HCl buffer (pH 7.4)–3.3mM-$MgCl_2$. All operations were carried out at 4°C. Activity is expressed as nmol of ^{32}P incorporated in 20min. The number of experiments is given in parentheses.

Nuclear fraction	Activity (per whole fraction) (total)	(%)	(per mg of protein)	(per mg of DNA)
Control (1)				
Nuclear suspension	116	100	—	6.6
Chromatin	45	39	—	2.9
Saline extract	106	91	8.5	—
Sham-operated (2)				
Nuclear suspension	133	100	—	3.6
Chromatin	32	24	—	3.2
Saline extract	114	86	3.8	—
Regenerating (2)				
Nuclear suspension	940	100	—	35
Chromatin	78	8	—	4.5
Saline extract	690	73	19.0	—

tributed about 8% of the substrate present in these assays.

Centrifugation of the nuclear gel ('suspension') at 25000*g* for 20min to sediment nuclear membranes and nucleoli gave very little loss in enzyme, indicating that the activity was associated with chromatin. When the preparations were diluted so that nucleohistone was precipitated, up to 90% of the activity remained with non-histone proteins in the sodium chloride extract. This fraction also showed markedly greater activity in nuclei from regenerating liver. Good recoveries of enzyme activity were found between nuclear subfractions. If the sodium chloride concentration was increased to 0.25–0.5M the chromatin-bound histone phosphokinase was 63–91% inhibited although a number of nuclear enzymes may be activated under these conditions (cf. Widnell & Tata, 1964).

Experiments in which fresh liver was homogenized in 0.25M-sucrose and centrifuged at 105000*g* for 60min showed no differences in amount of soluble enzyme from the liver supernatants centrifuged at 20000*g*. The histone phosphokinase was therefore not bound on to microsomes. The

Table 4. *Effects of 3′:5′-cyclic AMP on histone phosphokinase activity in rat liver cell fractions*

The activation factor is specific activity in the presence of cyclic AMP, divided by specific activity in its absence. The number of experiments and range of results are given in parentheses.

Cell fraction	Activation factor
Non-aqueous preparations	
Solvent-treated parencyhmal powder, control rat livers (1)	4.5
Nuclear suspensions and residues, control and regenerating rat livers (4)	1.2 (0·99–1·56)
Sucrose preparations	
Nuclear suspension, chromatin and saline extract, control rat livers (7)	1.4 (1·2–1·53)

Table 5. *Comparison of histone phosphokinase activity for histones F1, F2a and F3 in non-aqueous cell fractions from rat liver*

Activities are given as nmol of ^{32}P incorporated/20 min per mg dry weight.

Cell fraction	Activity			F1/F2a	F1/F3	F3/F2a
	F1	F2a	F3			
Control						
Parenchymal powder	0.33	0.065	0.28	5.1	1.2	4.3
Solvent-treated parenchymal powder	0.31	0.048	0.22	6.5	1.4	4.6
Nuclear suspension	0.64	0.072	0.19	9.4	3.4	2.6
Nuclear residue	0.59	0.039	0.19	15	3.1	4.9
Regenerating						
Parenchymal powder	0.30	0.085	0.21	3.5	1.4	2.5
Solvent-treated parenchymal powder	0.24	0.050	0.18	4.8	1.3	3.6
Nuclear suspension	0.37	0.019	0.08	18	4.9	4.0
Nuclear residue	0.22	0.014	0.06	16	3.9	3.9

specific activity of the cytoplasmic enzyme 22h after regeneration was about 25% of that extractable from the nuclei in dilute sodium chloride (Pawse *et al.* 1971). The high activity of enzyme found in nuclei from regenerating livers was therefore probably not due to simple retention in the nuclei of soluble enzyme of the cytoplasm.

Langan (1968) found about fivefold activation of the kinase for histone F1 by 0.1 μM-3′:5′-cyclic AMP. Comparable stimulation occurs with soluble enzyme extracted from solvent-treated parenchymal powder (Table 4), but neither in the nuclei isolated by the non-aqueous technique nor in those prepared in hyperosmotic sucrose was the bound enzyme appreciably activated by cyclic AMP. The bound enzyme from non-aqueous nuclei of regenerating livers was also relatively unaffected.

A preliminary experiment was performed to determine the capacity of the cell fractions (non-aqueous procedure) to phosphorylate histone F2a and histone F3 (Table 5). ^{32}P was transferred into these histones by both cytoplasm and nuclei although histone F2a was a poor phosphate acceptor (see also Langan & Smith, 1967). The results suggest that the bound enzyme in the nucleus was relatively more active towards histone F1 than was the soluble enzyme of the cytoplasm. Histones F3 and F2a were phosphorylated in almost the same proportions by all the cell fractions examined.

A further enzyme associated with histone phosphorylation is the histone phosphatase described in liver by Meisler & Langan (1969); its activity was optimum in 0.1–0.2 M-sodium chloride. The enzyme was found by them in the soluble fraction of the cytoplasm and in nuclei. Liver-cell fractions prepared by the non-aqueous procedure were therefore studied; 6–10 nmol of ^{32}P was released from histone F1/20 min per mg of protein by the solvent-treated parenchymal powder from normal liver. A similar order of activity was found for enzyme bound on the nucleus. The conditions of assay were chosen to give maximum amounts of activity (Meisler & Langan, 1969); they therefore contrast sharply with the conditions used for measurements of the histone kinase. The activity of the kinase in all the experiments presented here is based on nmol

of ^{32}P transferred. Since the preparations contained active histone phosphatase the extent of net phosphorylation catalysed by the kinase cannot be determined precisely, but from the rate of ^{32}P incorporation into regenerating liver 20% of histone F1 in the nucleus could take up one phosphate group/min.

DISCUSSION

An enzyme concerned with another modification of histone microstructure, acetylation, has also been located in cell nuclei (Allfrey, 1970; Gallwitz, 1970; Bondy, Roberts & Morelos, 1970). The presence of histone phosphokinase in the nucleus raises two problems: the roles of the soluble and nuclear forms of the phosphokinase and the identity of the two enzymes. Phosphorylation of histone F1 occurs during the synthetic period of the cell cycle. Both the soluble form of the enzyme (Pawse *et al.* 1971) and that bound to the nucleus increase during this period in regenerating liver. At present it is impossible to distinguish the contribution that either form might make to the increase in phosphate content found in histone F1 in the S period. Further, the results do not allow us to decide whether the soluble and nuclear forms of the enzyme are identical; the lack of activation of the nuclear enzyme by cyclic AMP may be associated with its bound state.

Histone F1 phosphatase has also been located in the chromatin fraction of the nucleus. At present the part played by alterations in activities of this nuclear enzyme in the overall balance of phosphorylation of histone F1 is unknown. The activity of the enzyme in the cytosol, when assayed under the different conditions used for histone kinase in their work (Pawse *et al.* 1971) did not alter during the first 22h of liver regeneration.

Cell fractions obtained by non-aqueous procedures were also able to phosphorylate histones F2a and F3. There is some suggestion that the relative activities of nuclei with respect to the three histones used in these studies differ from those found with cytoplasmic material. The significance of this requires further investigation.

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