Purification of non-histone acceptor proteins for ADP-ribose from mouse testis nuclei

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Acceptor proteins for poly(ADP-ribose) have been purified from mouse testis nuclei. Nuclear proteins were labelled *in vitro* with [¹⁴C]ribose and [³H]adenine, extracted with 5% (v/v) HClO₄ and 0.25M-HCl and separated by ion-exchange chromatography. Non-histone proteins were found to be the major acceptors in both the 5% (w/v)-HClO₄-soluble and 5%-HClO₄-insoluble HCl-extractable fractions. Of the two groups of non-histone proteins associated with chromatin, the LMG (low-mobility-group) proteins were preferentially ADP-ribosylated. HMG (high-mobility group) proteins were labelled to lower specific radioactivity. Six LMG proteins were purified to approx. 90% homogeneity and were identified from their mobility on polyacrylamide gels at pH 2.9 and from their amino acid composition. The average length of the poly(ADP-ribose) chain was estimated to be four to six repeating ADP-ribose units. It is suggested that ADP-ribosylation of LMG proteins, a long-neglected group of chromatin-associated proteins, is important during spermatogenesis for the production of spermatozoa with intact and competent DNA.

The major group of chromatin-associated proteins in eukaryotic cells, the histones, are generally regarded as having a structural role or at best act as 'blanket' repressors of gene transcription (Elgin & Weintraub, 1975). Recently, interest has developed in the non-histone chromosomal proteins, known as the LMG and HMG proteins (Goodwin *et al.*, 1973), as possible regulators of chromatin activity. HMG proteins have been investigated in considerable detail (reviewed by Johns, 1982) and have been found in all mammalian tissues examined so far with the exception of spermatozoa (Kennedy & Davies, 1980; Brown & Goodwin, 1983). The levels of HMG proteins 1 and 2 have been shown to vary significantly, not only between different tissues (Gordon *et al.*, 1980; Mathew *et al.*, 1981), but also between different physiological states of the same tissue (Seyedin & Kistler, 1979). HMG proteins 14 and 17 have been found to be

localized in DNAase-I-sensitive nucleosomes (Levy-Wilson *et al.*, 1977; Vidali *et al.*, 1978; Weisbrod & Weintraub, 1979), suggesting that they may be involved in maintaining chromatin in a transcriptionally competent form. Therefore of particular interest are processes that modify the properties of these proteins, such as ADP-ribosylation, since this might correlate with functional changes. The enzymic transfer of ADP-ribose from NAD to nuclear proteins is well known and has been correlated with a variety of chromatin-directed cellular events (reviewed by Hayaishi & Ueda, 1977). One current view is that ADP-ribosylated proteins function in some way to ensure efficient repair of DNA sequences undergoing transcription or replication (Faraneh *et al.*, 1982).

In previous work we have shown that mammalian testis contains a very active poly(ADP-ribose) synthetase (Farina *et al.*, 1979) and that the major acceptor proteins for ADP-ribose *in vivo* are non-histone proteins (Faraone Mennella *et al.*, 1982). Two ADP-ribosylated proteins were isolated from HCl extracts of mouse testis nuclei and were found to have the characteristics of HMG and LMG

Abbreviations used: HMG proteins, high-mobility-group proteins; LMG proteins, low-mobility-group proteins; CM-cellulose, carboxymethyl-cellulose; SDS, sodium dodecyl sulphate; DNAase, deoxyribonuclease.

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proteins. However, in the course of this work we observed that other ADP-ribose-labelled proteins were present in the HClO_4 - and HCl -soluble extracts. In the present investigation we carried out a more extensive purification and analysis of nuclear acceptor proteins for ADP-ribose in mouse testis nuclei with a view to understanding the importance of this process for spermatogenesis, DNA replication and cell proliferation.

Materials and methods

Materials

All chemicals and enzymes were of the highest purity available commercially and were obtained from Sigma or BDH. Amersham International supplied $\text{D-[1-}^{14}\text{C]ribose}$ (60 Ci/mol) and $[8\text{-}^3\text{H}]\text{-adenine}$ (15–25 Ci/mol). M_r marker proteins, DEAE-Sephadex A-25 and CM-Sephadex C-25 were purchased from Pharmacia, CM-cellulose 32 from Whatman, and Bio-Rex 70 and AG I-X2 resins (200–400 mesh, 2% cross-linkage) from Bio-Rad Laboratories. T.l.c. plates pre-coated with silica-gel G (1 mm) were obtained from Merck. Calf thymus glands were frozen immediately after collection and extracted with mineral acids as described below for testis nuclei.

Extraction and analysis of labelled proteins from testis nuclei

Adult male mice (CFLP strain) weighing 30–40 g were anaesthetized with diethyl ether and injected intra-testicularly with $5\text{ }\mu\text{Ci}$ of $[^{14}\text{C}]\text{ribose}$ and $5\text{ }\mu\text{Ci}$ of $[^3\text{H}]\text{adenine}$. After 2 h, animals were killed, the testes removed and nuclei prepared as described by Utakoji *et al.* (1968). Preliminary experiments showed that maximum incorporation of radiolabel occurred after 2 h (Faraone Mennella *et al.*, 1982). Phenylmethanesulphonyl fluoride (1 mM) was included in all nuclei-preparation buffers. Nuclei were homogenized in 3 vol. of ice-cold 5% (v/v) HClO_4 and centrifuged at 10000 g for 20 min. Precipitated proteins were washed three times in 5% HClO_4 at 4°C and then extracted with 0.25 M-HCl as described by Ueda *et al.* (1975). The 5%- HClO_4 washes were pooled with the original 5%- HClO_4 supernatant. The HCl-extracted proteins were chromatographed on a column (0.9 cm $\times$ 12.5 cm) of CM-cellulose as described by Ueda *et al.* (1975), whereas the HClO_4 -soluble proteins were analysed by the same procedure but with a gradient of NaCl from 0 to 0.42 M in 5 mM-acetate buffer, pH 4.2. Further chromatography of the first peak (designated 'PC1') from CM-cellulose 32 of the HCl-extracted proteins was carried out on a column (0.9 cm $\times$ 15.10 cm) of CM-Sephadex C-25, essentially as described by Rabbani *et al.* (1980), but at a lower pH (7.7) in order to lessen the

breakdown of protein-ADP-ribose bonds. The first protein fraction (designated 'PS1') was precipitated with 2% (w/v) trichloroacetic acid, and the centrifuged (10000 g for 20 min) pellet chromatographed on a column (1.5 cm $\times$ 18 cm) of DEAE-Sephadex A-25 in 0.075 M-Tris/HCl buffer, pH 7.4, with a gradient of NaCl from 0 to 1.0 M. The trichloroacetic acid supernatant was mixed with 0.1 vol. of 2 M-HCl and proteins precipitated by the addition of 3 vol. of ice-cold acetone (Goodwin *et al.*, 1973). After centrifugation at 13000 g for 30 min, the acetone-soluble proteins were chromatographed on a column (0.9 cm $\times$ 6.0 cm) of Bio-Rex 70 in 0.05 M-phosphate buffer, pH 6.5, containing 0.4 M-guanidinium chloride, followed by a gradient of 0.4–1.5 M-guanidinium chloride.

Electrophoresis

Proteins were analysed by electrophoresis on 15% (w/v)-polyacrylamide slab gels (11 cm $\times$ 13 cm or 6 cm $\times$ 8 cm) at pH 2.9 (Goodwin *et al.*, 1973). Estimates of apparent M_r were made by electrophoresing proteins at pH 2.9 in the first dimension, followed by their separation on polyacrylamide gels containing 0.4% (w/v) SDS in the second dimension (Jones *et al.*, 1980). Proteins were stained with 0.05% (w/v) Coomassie Blue R-250 in methanol/acetic acid/water (40:7:53, by vol.), followed by destaining in the same solvent without dye. Radioactivity in gel slices and in aqueous samples was measured by solubilization in 5 ml of Lumagel scintillation fluid.

Amino acid analysis

Purified proteins were hydrolysed in 6 M-HCl at 110°C for 20 or 70 h and released amino acids were analysed on a Beckman (model 119) amino acid analyser. Amino acid analysis was also performed on gel slices containing proteins as described by Levy-Wilson *et al.* (1979).

Digestion with snake-venom phosphodiesterase and analysis of products

Fractions to be analysed were first neutralized with KOH and incubated for 48 h at 25°C with 100 μg of phosphodiesterase/ml in 0.25 M-Tris/acetate buffer, pH 8.8, containing 0.025 mM- MgCl_2 . The digestion was terminated by the addition of HClO_4 to 5%, the mixture centrifuged at 15000 g for 30 min and the supernatant chromatographed on AG1-X2 (formate form) resin column (Ueda *et al.*, 1975). Peaks containing radioactivity were analysed further by t.l.c. in silica-gel G in isobutyric (2-methylpropanoic) acid/ NH_3 /water (66:1:33, by vol.) solvent system. Known standards of 5'-AMP and ADP-ribose were chromatographed under identical conditions.

Other procedures

Total protein was measured by a modified Lowry procedure (Bensadoun & Weinstein, 1976), with bovine serum albumin as standard.

Results

The chromatographic procedure for separating non-histone proteins and the radioactivity recovered in each fraction are summarized in Scheme 1. Approx. 3.7 mg of protein was present in a 10000 g centrifuged supernatant after homogenizing nuclei prepared from 20 g of testes in 5% HClO_4 . This HClO_4 extract had a specific radioactivity of 104000 c.p.m./mg, and when analysed on 15% (w/v)-polyacrylamide gels at pH 2.9 contained a complex mixture of proteins consisting mainly of histone H1 together with a variety of LMG- and HMG-like proteins as defined by Goodwin *et al.* (1973) (Fig. 1). The HClO_4 -insoluble HCl -extractable fraction, on the other hand, contained 30.6 mg of protein (specific radioactivity 13000 c.p.m./mg) and consisted mostly of histones H2a, H2b, H3 and H4, with trace amounts of H1 (Fig. 1). This fraction was greatly enriched in LMG-like proteins.

Comparison of testicular non-histone proteins with calf thymus HMG proteins

To aid identification of testicular non-histone proteins and to provide a reference point with

published work, a preliminary investigation was made of HMG proteins from calf thymus tissue extracted and analysed by the same procedures as described for testis. Calf thymus HMG 2, 14, 17 and 20 (ubiquitin) were identified from their mobility on polyacrylamide gels at pH 2.9 (Fig. 2) and from their amino acid composition (Table 1). The results are very close to those reported by Johns and co-workers (summarized by Mayes & Johns, 1982). Table 1 also shows the amino acid composition of testicular HMG 2, 14, 17 and 20, which were identified as having the same mobility on polyacrylamide gels at pH 2.9 as their calf thymus counterparts (Fig. 2). The results are very similar to those found for calf thymus HMG proteins, with characteristically high levels of basic and acidic amino acids. We therefore consider that the analytical procedures employed in the present investigation are comparable with those used by other workers.

Analysis of labelled proteins in 5%- HClO_4 extracts of testis nuclei

When the HClO_4 -soluble proteins were separated by chromatography on CM-cellulose 32, approx. 60% of the total radioactivity applied was recovered in the first protein peak (designated 'SC1' Fig. 3) with a specific radioactivity of 1.3×10^6 c.p.m./mg. Three other major radioactive peaks, designated 'SC2', 'SC3' and 'SC4', were

Fig. 1. Electrophoresis on a 15%-polyacrylamide gel at pH 2.9 (Goodwin *et al.*, 1973) of acid-soluble proteins from mouse testis nuclei

Track (i), total HClO_4 -insoluble HCl -extractable proteins (100 μg); track (ii), total HClO_4 -soluble proteins; track (iii), fraction SC1 (10 μg) from Fig. 3; track (iv), fractions SC2 (5 μg) and SC3 (25 μg) from Fig. 3. The mobilities of a mixture of calf thymus histone standards are shown for reference.

Fig. 2. Electrophoresis of proteins extracted from calf thymus and mouse testis with 5% HClO_4 on a 15%-polyacrylamide slab gel at pH 2.9

Track (i), testis (50 μg); track (ii), calf thymus (50 μg). The mobilities of HMG-1, -2, -14, -17 and -20 are indicated. Arrows identify proteins SC1, SC2, SC3 and histone H1.

Scheme 1. Summary of procedures used to purify labelled HMG- and LMG-like proteins from mouse testis nuclei. The values given refer to the total protein and total radioactivity recovered in each fraction.

Table 1. *Amino acid analysis of HMG-2, -14, -17 and -20 from calf thymus and mouse testis*

Amino acid	Amino acid composition (mol/100 mol)															
	HMG-2				HMG-14				HMG-17				HMG-20			
	Calf thymus*	Calf thymus	Mouse testis		Calf thymus†	Calf thymus	Mouse testis		Calf thymus†	Calf thymus	Mouse testis		Calf thymus†	Calf thymus	Mouse testis	
Lysine	19.4	19.5	19.3		21.1	20.1	20.0		24.3	23.5	24.1		9.2	9.6	10.6	
Histidine	2.0	1.9	2.7		0.2	0.6	0.5		—	Trace	0.6		1.3	1.7	0.9	
Arginine	4.7	4.3	4.0		5.4	5.4	5.8		4.1	3.5	3.3		5.5	5.0	4.8	
Aspartic acid	9.3	9.7	10.0		8.3	9.8	9.0		12.0	11.6	13.0		9.7	9.8	8.9	
Threonine	2.7	3.3	2.7		4.1	5.0	4.6		1.2	1.7	1.1		9.0	8.9	9.6	
Serine	7.4	7.4	10.0		8.0	8.3	8.3		2.3	2.4	2.6		4.4	4.9	4.8	
Glutamic acid	17.5	17.3	16.6		17.5	17.1	16.0		10.5	10.2	11.0		16.5	16.7	16.1	
Proline	8.9	8.3	8.7		8.1	9.0	8.1		12.9	11.2	10.2		4.6	4.6	4.2	
Glycine	6.5	6.2	7.3		6.4	6.5	5.8		11.2	13.5	11.2		5.8	6.1	6.5	
Alanine	8.1	8.6	8.8		14.8	14.5	13.6		18.4	17.5	17.6		2.9	3.1	3.0	
Valine	2.3	3.8	3.0		4.0	4.1	4.4		2.0	2.9	2.1		5.4	5.6	6.5	
Methionine	0.4	Trace	Trace		0.1	0.5	Trace		—	0.6	Trace		1.2	Trace	1.6	
Isoleucine	1.3	2.0	2.0		0.3	0.8	0.3		—	0.6	1.0		9.0	9.0	7.6	
Leucine	2.0	3.0	3.3		2.0	2.7	2.4		1.0	1.1	1.3		12.4	11.4	11.8	
Tyrosine	2.0	2.9	0.6		0.2	0.2	0.3		—	Trace	0.3		1.3	1.6	1.4	
Phenylalanine	3.0	3.3	0.7		0.3	0.3	0.4		—	Trace	0.4		2.7	2.9	2.3	
Lysine/arginine	4.1	4.5	4.8		3.9	3.7	3.5		6.1	6.7	7.3		1.7	1.9	2.1	
Basic amino acids (%)	26.1	25.7	26.0		26.7	23.1	26.4		28.3	27.0	28.0		16.0	16.3	15.9	
Acidic amino acids (%)	26.8	26.9	26.6		25.8	26.9	25.0		22.5	21.7	24.0		26.2	25.6	25.0	
Acidic/basic amino acid ratio	1.02	1.04	1.02		0.97	1.02	0.94		0.79	0.80	0.85		1.63	1.56	1.57	

* From Goodwin *et al.* (1978).

† From Walker (1982).

† From Schlesinger *et al.* (1975).

eluted with the NaCl gradient (Fig. 3). An analysis of fractions on polyacrylamide gels at pH 2.9 indicated that SC4 contained mostly H1 histone

Fig. 3. Chromatography on CM-cellulose 32 of HClO_4 -soluble proteins from mouse testis nuclei labelled *in vivo* with [^{14}C]ribose and [^3H]adenine

Proteins were applied to the column in 5mM-acetate buffer, pH 4.2, followed by elution with a linear gradient (●) of 0–0.42M-NaCl. Protein was measured by A_{230} (—) and total radioactivity (---) by counting portions from each fraction in 5ml of Lumagel scintillation fluid in a Beckman liquid-scintillation counter (model LS8100).

(results not shown), but that SC1, SC2 and SC3 were composed of proteins with low (SC1) and high (SC2 and SC3) mobility (Fig. 1). In a typical experiment the relative amounts of radioactivity recovered from gel slices containing these proteins were 7280, 450 and 600 c.p.m. for SC1, SC2 and SC3 respectively. Electrophoresis of these proteins in the second dimension on a 15%-polyacrylamide gel containing SDS showed that SC1 migrated with an M_r of 16000, SC2 with an M_r of 10500 and SC3 with an M_r of 11500 (results not shown). Identification of SC1 as an LMG-like protein was supported by its high content of acidic amino acids (35%) and its low content of basic amino acids (7.5%), particularly lysine (Table 2). On the other hand, the amino acid compositions of SC2 and SC3 resembled those of HMG-like proteins, as indicated by the high percentage of both acidic (22–24%) and basic (23%) residues (Table 2). Although proteins SC2 and SC3 resemble HMG 14, 17 and 20, they still exhibit significant differences with respect to electrophoretic mobility and composition, the most prominent of which is the lower content of proline residues. We must also add that although the highest levels of radioactivity were always in proteins SC1, SC2 and SC3, in some experiments significant radioactivity was also found in protein bands corresponding to HMG-14 and -17, with trace amounts in HMG-20.

Table 2. Amino acid composition of ADP-ribosylated non-histone acceptor proteins purified from mouse testis nuclei. Non-histone acceptor proteins for ADP-ribose were extracted and purified as described in the Materials and methods section. Values for aspartic acid and glutamic acid include asparagine and glutamine respectively. Abbreviation used: ND, non-detectable.

Amino acid	Protein	...	Composition (mol/100mol)							
			SC1	SC2	SC3	PB1	PA	PD6	PD8	PD9
Lysine			4.6	17.2	17.1	4.7	5.8	4.6	4.0	5.0
Histidine			1.6	1.8	1.5	1.7	1.4	2.2	2.0	2.1
Arginine			1.3	4.1	4.6	2.2	5.3	1.3	1.8	1.4
Aspartic acid			12.0	10.6	9.5	13.8	11.9	9.2	10.2	11.5
Threonine			5.4	5.2	7.3	5.2	4.7	1.8	3.7	2.9
Serine			10.8	8.7	7.0	10.9	11.0	12.2	12.5	8.4
Glutamic acid			23.3	11.1	14.5	11.6	10.6	17.9	17.0	16.4
Proline			2.6	2.0	2.1	ND	ND	ND	ND	ND
Glycine			16.0	12.3	13.1	17.5	14.5	24.0	24.5	23.0
Alanine			9.8	9.1	5.1	10.4	13.3	9.2	7.4	9.1
Cysteine			ND	ND	ND	ND	ND	ND	ND	ND
Valine			6.1	4.8	5.5	5.8	5.7	4.5	4.7	6.0
Methionine			0.9	1.0	1.6	1.3	1.3	1.5	0.6	1.2
Isoleucine			1.9	4.0	3.2	3.4	2.6	2.8	2.8	3.4
Leucine			3.1	7.0	4.6	6.5	5.7	4.5	4.8	6.0
Tyrosine			0.6	1.3	0.9	2.1	1.9	2.5	1.5	1.2
Phenylalanine			1.1	1.7	2.4	2.7	2.3	1.7	2.0	1.9
Lysine/arginine	...		3.5	4.2	3.7	2.1	1.1	3.5	2.2	3.5
Basic amino acids (%)	...		7.5	23.1	23.2	8.6	12.5	8.1	7.8	8.5
Acidic amino acids (%)	...		35.3	21.7	24.0	25.4	23.0	27.0	27.9	27.9
Acidic/basic amino acid ratio	...		4.7	0.9	1.0	2.9	1.8	3.3	3.5	3.3
$10^{-3} \times M_r$	...		16.0	10.5	11.5	29.0	68.0	58.0	61.0	60.0

Analysis of labelled proteins in the HClO_4 -insoluble HCl -extractable fraction

In agreement with our previous results (Faraone Mennella *et al.*, 1982), we found that, after chromatography on CM-cellulose 32, approx. 90% of the total radioactivity in the 0.25M-HCl extract was associated with non-histone proteins (fraction PC1; Fig. 4). The remaining labelled proteins were eluted in fractions PC2, PC3 and PC4, which, when analysed on polyacrylamide gels at pH 2.9, each contained one major protein with the same mobility as histones H1, H2a+H2b and H3 respectively (results not shown). These fractions were not investigated further. When fraction PC1 (containing 7.8mg of protein, specific radioactivity 23000 c.p.m./mg) was chromatographed on CM-Sephadex C-25 to separate LMG-like and HMG-like proteins (Rabbani *et al.*, 1980), only about 10% of the total protein bound to the column (Fig. 5). This column was subsequently eluted with a gradient of NaCl to yield three major labelled protein fractions, PS2, PS3 and PS4. This behaviour is characteristic of HMG-like proteins (Rabbani *et al.*, 1980). Analysis of these fractions on polyacrylamide gels at pH 2.9 confirmed this conclusion, revealing that the major proteins had

mobilities greater than that of histone H₄ (results not shown).

Fraction PS1, containing approx. 92% of the labelled proteins, was made 2% (w/v) with respect to trichloroacetic acid, the precipitated protein removed by centrifugation and solubilized in 0.075M-Tris/HCl buffer, pH 7.4. Chromatography on DEAE-Sephadex A-25 yielded three major labelled protein fractions (PD6, PD8 and PD9) (Fig. 6). Analysis of the proteins in PD6, PD8 and PD9 on polyacrylamide gels at pH 2.9 showed one major protein in each fraction (Fig. 7), all with very

Fig. 4. Chromatography on CM-cellulose 32 of HClO_4 -insoluble HCl -extractable proteins from mouse testis nuclei labelled *in vivo* with [^{14}C]ribose and [^3H]adenine

Proteins were applied to the column in 0.1M-sodium acetate buffer, pH 4.2, followed by stepwise elution with 0.17M-acetate buffer, pH 4.2, containing (A) 0.42M-NaCl, (B) 0.01M-HCl and (C) 0.02M-HCl respectively as indicated by the arrows. The column was calibrated with known histone standards (H1 *et.*), whose elution volumes are indicated on the abscissa. Protein was measured by A_{230} (—) and total radioactivity (----) as described in Fig. 3.

Fig. 5. Chromatography on CM-Sephadex C-25 of the PC1 fraction eluted from CM-cellulose 32 (see Fig. 4) After dialysis against 7.5mM-sodium borate buffer, pH 7.7, proteins were applied to the column in the same buffer, and bound material was eluted with a linear gradient (●) of 0.15–1.25M-NaCl in the same buffer. Protein was measured at A_{230} (—) and radioactivity (----) as described in Fig. 3.

Fig. 6. Chromatography on DEAE-Sephadex A-25 of the trichloroacetic acid-insoluble material from fraction PS1 (Fig. 5)

Proteins were applied to the column in 7.5mM-Tris/HCl, pH 7.4, and bound material was eluted with a linear gradient (●) of 0–1.0M-NaCl in the same buffer. Protein (A_{230} , —) and radioactivity (----) were monitored in fractions as described in Fig. 3.

Fig. 7. Electrophoresis on a polyacrylamide slab gel (pH 2.9) of proteins in fractions PD6, PD8, PD9, PA and PB1

Track (i), fraction PA (3 µg); track (ii), total HCl extract (50 µg); track (iii), fraction PB1 (3 µg); track (iv), fraction PD8 (4 µg); track (v), fraction PD9 (4 µg); track (vi), fraction PD6 (3 µg). The mobilities of histone standards are shown for reference.

low mobility. M_r estimates from SDS/polyacrylamide gels were 58 000, 61 000 and 60 000 for PD6, PD8 and PD9 respectively. Amino acid analysis of each of these three proteins revealed a low content of basic amino acids (7.5–8.5%), particularly lysine residues, with an acidic/basic amino acid ratio of >3.0 (Table 2).

The 2% (w/v)-trichloroacetic acid supernatant (containing 80–90% of the radioactivity in fraction PS1) was mixed with 3 vol. of acetone, and precipitated proteins (PA) analysed on polyacrylamide gels at pH 2.9. A single major protein with low mobility was observed (Fig. 7). This protein had an M_r of 68 000 on SDS/polyacrylamide gels, a lysine/arginine ratio of 1:1, and a low content of basic amino acids (12.5%, Table 2). Proteins in the acetone-soluble fraction were chromatographed on a column of Bio-Rex 70 (Fig. 8), which yielded a single major labelled protein peak (PB1). This peak contained one protein when analysed on polyacrylamide gels at pH 2.9 (Fig. 7), with a slightly lower mobility than H1 histone. Its M_r was 29 000 and the acidic/basic amino acid ratio was 2.9 (Table 2).

Characterization of ADP-ribose derivatives released by snake-venom phosphodiesterase

To verify that the radioactivity present in the HClO_4 - and HCl-extracts and various protein fractions was in the form of covalently bound ADP-ribose, samples were digested with snake-venom phosphodiesterase, and acid-soluble products were analysed on a AG1-X2 resin (formate form) column. A typical example is shown in Fig. 9 for protein SC1. In this case the enzymic digestion

Fig. 8. Chromatography of trichloroacetic acid-soluble acetone-soluble proteins from fraction PS1 on Bio-Rex 70

Proteins were applied in 50 mM-phosphate buffer, pH 6.5, containing 0.4 M-guanidinium chloride, and bound material was eluted by a linear gradient (●) of 0.4 M–1.5 M-guanidinium chloride in phosphate buffer. Protein (A_{230} , —) and radioactivity (----) were monitored as described in Fig. 3.

released more than 80% of the bound radiolabel and the major products co-chromatographed with authentic 5'-AMP and ADP-ribose standards on an AG1-X2 resin (formate form) column. The identity of these reaction products was confirmed by further analysis on silica-gel G plates (results not shown). From radioactivity recovered in the fractions corresponding to 5'-AMP and iso-ADP-ribose for the SC1 protein, the ADP-ribose chain length was estimated to be four to six repeating ADP-ribose units (Hayaishi & Ueda, 1977). In addition to this procedure, ADP-ribosylation of the other protein samples was checked by chromatography on AG1-X2 resin (formate form) columns of deproteinized alkaline hydrolysates. In each case a radioactive peak was obtained that was identified as ADP-ribose.

Discussion

The results of this investigation indicate that, in mouse testis nuclei, non-histone proteins are the major acceptors for poly(ADP-ribose) and that, of the two major groups of non-histone proteins present in the nucleus, the LMG proteins are preferentially ADP-ribosylated.

Since their discovery by Goodwin and co-workers in 1973 (Goodwin *et al.*, 1973), relatively

Fig. 9. Chromatography of labelled products released from fraction SC1 (Fig. 2) after digestion with snake-venom phosphodiesterase on an AG-1-X2 (formate form) resin column

Proteins were eluted with step gradients (●) of: A, water; B, water to 0.3M-formic acid; C, 0.3–4M-formic acid; D, 4M-formic acid–6M-formic acid + 0.6M-ammonium acetate. Values for A_{260} (—) indicate the elution profile of authentic 5'-AMP and ADP-ribose (ADPR) standards. Radioactivity (----) was monitored as described in Fig. 3.

little attention has been paid to LMG proteins, although a lot of interest has centred on the purification, structure and organization of HMG proteins in chromatin (Johns, 1982). Our classification of proteins SC1, PB1, PA, PD6, PD8 and PD9 as LMG proteins is based on their poor ability to penetrate 15% polyacrylamide gels at pH 2.9 [the same gel system used originally by Goodwin *et al.* (1973)] and their low content of basic amino acids, particularly lysine residues. Under the same conditions, HMG proteins would have a higher mobility (e.g. SC2 and SC3) and have high levels of both basic and acidic amino acids (Table 2). Furthermore, HMG proteins generally have a lower M_r than LMG proteins.

It is difficult to draw comparisons with other work on the composition of LMG proteins as little information is available in the literature, but the present results compare favourably with those published by Goodwin *et al.* (1973) for total LMG proteins extracted from calf thymus. The main point of difference is the lower concentration of proline found in mouse LMG proteins, although this may be a species variation. Phylogenetic

differences have been noted for HMG proteins (Christensen & Dixon, 1981; Brown & Goodwin, 1983). Among the LMG-like proteins we have isolated, it is noticeable that proteins PD6, PD8 and PD9 have a similar amino acid composition, M_r and electrophoretic mobility on polyacrylamide gels at pH 2.9. It is possible that they represent a family of closely related proteins with just sufficient variation between them to give different elution volumes from DEAE-Sephadex. Precedents for this exist in that microheterogeneity has been reported in HMG proteins from trout testis (Brown *et al.*, 1980) and calf thymus (Cooper & Spaulding, 1983). PD6, PD8 and PD9 are unlikely to be proteolytic degradation products of histones or HMG proteins because they have such low mobility on both SDS/polyacrylamide gels and acid polyacrylamide gels at pH 2.9. There is no evidence that ADP-ribosylated histones or HMG proteins are less stable than their LMG counterparts, so it would seem unlikely that preferential degradation could account for the unequal distribution of radioactivity between the different protein fractions. Rather, the evidence points

towards a difference in available acceptor substrates to the poly(ADP-ribose) synthetase.

Of all the ADP-ribosylated proteins extracted from testis nuclei, protein SC1 was labelled to the highest specific radioactivity. We consider it unlikely that SC1 is a breakdown product of HMG-1 or -2 for the following reasons. (1) SC1 has a lower mobility on acid (pH 2.9) polyacrylamide gels than HMG-1 or -2. Proteinase digestion products of HMG1 and 2 have been found to have higher mobilities than has the parent molecule (Cary *et al.*, 1983). (2) SC1 has a very low percentage (7.5%) of basic amino acids, with an unusually high proportion of acidic residues (35%). Although HMG-1 and -2 have acidic amino acids concentrated in the C-terminal portion of the molecule (Walker, 1982), it is still difficult to envisage how a 16000- M_r fragment could be removed and retain the composition found for SC1; (3) there is some evidence that ADP-ribosylation inhibits proteinases (Inagaki *et al.*, 1980); (4) 5% HClO_4 is considered to be highly effective in inhibiting degradation of nuclear proteins (Minaga *et al.*, 1979). We therefore consider SC1 to be an LMG-like protein. Preliminary experiments indicate that protein SC1 is not specific to testis, but that various concentrations are present in other tissues as well (P. Quesada & R. Jones, unpublished work). Although SC1 is ADP-ribosylated to the highest specific radioactivity, it constitutes only approx. 1% of the total HClO_4 -soluble proteins from testis. This fact in itself suggests that it may have a regulatory function.

Protein PA is probably equivalent to the high- M_r protein we isolated previously from mouse testis (Faraone Mennella *et al.*, 1982). In several respects it resembles protein C-14 from Novikoff hepatoma ascites cells, particularly with regard to its M_r and amino acid composition (Orrick *et al.*, 1973; James *et al.*, 1977). Protein C-14 was found to bind avidly to DNA and to stimulate RNA synthesis (James *et al.*, 1977), and it is interesting to speculate that protein PA may have similar activity in mouse testis.

The HMG-like proteins SC2 and SC3 are very similar to mammalian HMG-14 and -17, the main points of divergence being the slight variation in their mobility on polyacrylamide gels at pH 2.9, their lower content of proline and alanine residues and their higher content of hydrophobic amino acids. Cooper & Spaulding (1983) have described three HClO_4 -soluble proteins (designated PS1, PS2 and PS3) in calf thymus and thyroid tissues whose electrophoretic properties resemble, but are significantly different from, those of HMG-14 and -17. They present strong evidence that PS1, PS2 and PS3 are not degradation products of HMG-14 and -17 but favour the view that they are variant forms

of the latter proteins. Brown & Goodwin (1983) have also isolated genuine variants of trout testis HMG-14 and -17. We consider it likely that a similar situation holds for SC2 and SC3.

The covalent linking of poly(ADP-ribose) to nuclear proteins has been described in a wide variety of tissues (reviewed by Hayaishi & Ueda, 1977) and has been implicated in DNA synthesis (Burzio & Koide, 1973), chromatin structure (Lorimer *et al.*, 1976), DNA excision-repair (Durkacz *et al.*, 1980; Levy-Wilson, 1981), gene expression (Muller & Zahn, 1976) and cell differentiation (Hilz & Stone, 1976; Faraneh *et al.*, 1982). It has been shown that poly(ADP-ribose) synthetases do not have a uniform distribution in chromatin, but are associated with DNAase I-sensitive, transcriptionally competent, nucleosomes (Mullins *et al.*, 1977; Butt *et al.*, 1978). ADP-ribosylated histones (Hayaishi & Ueda, 1977; Wong *et al.*, 1977; Okayama *et al.*, 1978) and non-histone proteins (Smith & Stocken, 1975; Okayama & Hayaishi, 1978; Levy-Wilson, 1981; Tanuma & Johnson, 1983) have been identified in nuclei and nucleosomes, implying that modification of chromatin-associated proteins is important in regulating the transcriptional activity of DNA. However, a direct correlation between ADP-ribosylation and induction or suppression of transcription has yet to be established, and present evidence suggests a role for ADP-ribosylation in DNA excision-repair and replication (Durkacz *et al.*, 1980; Nudka & Shall, 1980; Ohgushi *et al.*, 1980; Levy-Wilson, 1981). Since the testis shows a high rate of cell proliferation, it would be an obvious site for this kind of process. Seyedin & Kistler (1979) found a strong correlation between the levels of HMG-2 (but not HMG-1) and active spermatogenesis in rat testis, but did not investigate if HMG-2 was ADP-ribosylated or if ADP-ribosylation varied at different stages of spermatozoon development. It is known that histones and HMG (and presumably LMG) proteins are replaced by protamines during spermiogenesis (Mann, 1964; Loir & Lanneau, 1978), so it is to be expected that ADP-ribosylation would be diminished during the later stages of spermatozoon development. However, there is no information on the cellular distribution of poly(ADP-ribose) synthetases in the testis, and it is possible that much of the activity resides in Sertoli cells or Leydig cells rather than spermatogonia and primary spermatocytes. Since the testis is also a target organ for pituitary gonadotrophins, a role for ADP-ribosylation in gene expression and cell differentiation cannot be excluded.

In conclusion, we have shown that, in mouse testis nuclei, LMG proteins, a long-neglected group of chromatin-associated proteins, are prefer-

entially ADP-ribosylated, and we suggest that this process is important during spermatogenesis for the production of spermatozoa with intact and competent DNA.

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References

- Bensadoun, A. & Weinstein, D. (1976) *Anal. Biochem.* **70**, 241–250
- Brown, E. & Goodwin, G. H. (1983) *Biochem. J.* **215**, 531–538
- Brown, E., Goodwin, G. H., Mayes, E. L. V., Hastings, J. R. B. & Johns, E. W. (1980) *Biochem. J.* **191**, 661–664
- Burzio, L. & Koide, S. S. (1973) *Biochem. Biophys. Res. Commun.* **53**, 572–579
- Butt, T. R., Brothers, J. F., Giri, C. P. & Smulson, M. E. (1978) *Nucleic Acids Res.* **5**, 2775–2778
- Cary, P. D., Turner, C. H., Mayes, E. & Crane-Robinson, C. (1983) *Eur. J. Biochem.* **131**, 367–374
- Christensen, M. E. & Dixon, G. H. (1981) *J. Biol. Chem.* **256**, 7549–7556
- Cooper, E. & Spaulding, A. W. (1983) *Biochem. J.* **215**, 643–649
- Durkacz, B. W., Omidiji, O., Gray, D. A. & Shall, S. (1980) *Nature (London)* **283**, 593–596
- Elgin, S. C. R. & Weintraub, H. (1975) *Annu. Rev. Biochem.* **44**, 725–774
- Faraneh, F., Zalin, R., Brill, D. & Shall, S. (1982) *Nature (London)* **300**, 362–366
- Faraone Mennella, M. R., Quesada, P., Farina, B., Leone, E. & Jones, R. (1982) *Biochem. J.* **205**, 245–248
- Farina, B., Faraone Mennella, M. R. & Leone, E. (1979) in *Macromolecules in the Functioning Cell* (Salvatore, F., Marino, G. & Volpe, P., eds.), pp. 283–300, Plenum, New York
- Goodwin, G. H., Sanders, C. & Johns, E. W. (1973) *Eur. J. Biochem.* **38**, 14–19
- Goodwin, G. H., Walker, J. M. & Johns, E. W. (1978) in *The Cell Nucleus* (Busch, H., ed.), vol. 6, pp. 182–219, Academic Press, London and New York
- Gordon, J. S., Rosenfield, B. I., Kaufman, R. & Williams, D. L. (1980) *Biochemistry* **19**, 4395–4402
- Hayaishi, O. & Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116
- Hilz, H. & Stone, P. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58
- Inagaki, I., Miura, K. & Murachi, T. (1980) *J. Biol. Chem.* **255**, 7746–7750
- James, G. T., Yeoman, L. C., Matsui, S., Goldberg, A. H. & Busch, H. (1977) *Biochemistry* **16**, 2384–2389
- Johns, E. W. (1982) in *The HMG Chromosomal Proteins* (Johns, E. W., ed.), pp. 1–7, Academic Press, London and New York
- Jones, R., Brown, C. R., von Glos, K. I. & Parker, M. G. (1980) *Biochem. J.* **188**, 667–676
- Kennedy, B. P. & Davies, P. L. (1980) *J. Biol. Chem.* **255**, 2533–2539
- Levy-Wilson, B. (1981) *Arch. Biochem. Biophys.* **208**, 528–534
- Levy-Wilson, B., Wong, N. C. W. & Dixon, G. H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2810–2814
- Levy-Wilson, B., Watson, D. C. & Dixon, G. H. (1979) *Nucleic Acids Res.* **6**, 259–274
- Loir, M. & Lanneau, M. (1978) *Exp. Cell Res.* **115**, 231–243
- Lorimer, W. S., III, Stone, P. R. & Kidwell, W. R. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1624
- Mann, T. (1964) *The Biochemistry of Semen and of the Male Reproductive Tract*, pp. 139–160, Methuen, London
- Mathew, C. G. P., Goodwin, G. H., Igo-Kemenes, T. & Johns, E. W. (1981) *FEBS Lett.* **125**, 25–29
- Mayes, E. L. V. & Johns, E. W. (1982) in *The HMG Chromosomal Proteins* (Johns, E. W., ed.), chapter 10, pp. 223–247, Academic Press, London and New York
- Minaga, T., Romaschin, A. D., Kirsten, E. & Kun, E. (1979) *J. Biol. Chem.* **254**, 9663–9668
- Muller, W. E. C. & Zahn, R. K. (1976) *Mol. Cell Biochem.* **12**, 147–158
- Mullins, D. W., Jr., Giri, C. P. & Smulson, M. (1977) *Biochemistry* **16**, 506–513
- Nudka, N. & Shall, S. (1980) *Biochem. Biophys. Res. Commun.* **96**, 997–1002
- Ohgushi, H., Yoshihara, K. & Kamiva, T. (1980) *J. Biol. Chem.* **255**, 6205–6211
- Okayama, H. & Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun.* **84**, 755–762
- Okayama, H., Ueda, K. & Hayaishi, O. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1111–1115
- Orrick, L. R., Olson, M. O. J. & Busch, H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1316–1320
- Rabbani, A., Goodwin, G. H., Walker, J. M., Brown, E. & Johns, E. W. (1980) *FEBS Lett.* **109**, 294–298
- Schlesinger, D. L., Goldstein, G. & Niall, H. D. (1975) *Biochemistry* **14**, 2214–2218
- Seyedin, S. M. & Kistler, W. S. (1979) *J. Biol. Chem.* **254**, 11264–11271
- Smith, J. & Stocken, L. A. (1975) *Biochem. J.* **147**, 523–529
- Tanuma, S. & Johnson, G. S. (1983) *J. Biol. Chem.* **258**, 4067–4070
- Ueda, K., Omachi, A., Kawaichi, M. & Hayaishi, O. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 205–209
- Utakoji, T., Muramatsu, M. & Sugano, H. (1968) *Exp. Cell Res.* **53**, 447–458
- Vidali, G., Boffa, L. G., Bradbury, E. M. & Allfrey, V. G. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2239–2243
- Walker, J. M. (1982) in *The HMG Chromosomal Proteins* (Johns, E. W., ed.), pp. 69–87, Academic Press, London and New York
- Weisbrod, S. & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 630–634
- Wong, N. C. W., Poirier, G. G. & Dixon, G. H. (1977) *Eur. J. Biochem.* **77**, 11–21