The EMBO Journal vol.7 no.1 pp.57-67, 1988

At least 60 ADP-ribosylated variant histones are present in nuclei from dimethylsulfate-treated and untreated cells

Teni Boulikas

Département de Biochimie, Faculté de Médecine, Université de Sherbrooke, Fleurimont, Québec, Canada J1H 5N4

Communicated by B.Hirt

The level of histone adenosine diphospho (ADP) ribosylation was studied in isolated nuclei from mouse myeloma cells in culture. The cells were treated with dimethylsulfate (DMS), a DNA-methylating agent, and histones were analyzed using two-dimensional gel electrophoresis. Seventeen or more bands probably representing mono- to heptadeca (ADP-ribosylated) histones could be visualized for each major variant histone. DMS treatment, by increasing the number of chromatin sites undergoing repair, greatly enhanced histone ADP-ribosylation. When histones were labeled in a cell lysate rather than in isolated nuclei, mono- and oligo(ADP-ribosylated) histone forms prevailed. The presence of ~87 ADP-ribosylated variant histone forms in cell lysates and of ~170 in isolated nuclei is shown for the first time in this work. Previous studies show multiple ADP-ribosylated forms for only histone H1. The theoretical number of variegated nucleosomes is thus much higher than previously thought, provided that histone-histone contacts are not disrupted at up to a certain level of histone ADP-ribosylation.

Key words: poly(ADP-ribosylation)/DNA damage/chromatin/histones

Introduction

Adenosine diphospho (ADP) ribosylation is the most dramatic post-translational modification of histones and several other nuclear and cytoplasmic proteins (reviewed by Ueda and Hayaishi, 1985; Boulikas, 1987a). During this modification, ADP-ribose moieties from nicotinamide adenine dinucleotide (NAD^+) are enzymatically transferred to histones and several other proteins.

Figure 1 summarizes the chemical nature of poly(ADP-ribose) as well as the enzymes involved in its synthesis and degradation. The nuclear polymer is attached via the carboxyl group of the glutamic acid at position 2 to histone H2B (Burzio *et al.*, 1979) and via the γ-carboxyl groups of glutamic acid residues at positions 2, 14 and 116 and the α-carboxyl group of the C-terminal lysine residue at position 213 to histone H1 (Riquelme *et al.*, 1979; Ogata *et al.*, 1980a,b). The positions of ADP-ribosylation have not been determined for the other histones. Poly(ADP-ribose), as shown in Figure 1, contains a ribose α (1′-2′) ribose-phosphate-phosphate backbone (Miwa *et al.*, 1981). Branching every 30-50 residues involves the 2′ position of the ribose at the branch point whose 1′ position is involved in the linear ribose-ribose linkage (Miwa *et al.*, 1981).

Mono-modification of carboxyl groups (Kawaichi *et al.*, 1980), the subsequent elongation step of the polymer (Ueda *et al.*, 1979) as well as branching of the polymer by α (1′-2′) ribose-ribose linkages (Kawaishi *et al.*, 1981) are all carried out by the enzyme poly(ADP-ribose) synthetase or polymerase. The enzymes poly(ADP-ribose) glycohydrolase (Ueda *et al.*, 1972; Miwa *et al.*, 1974) and ADP-ribosyl protein lyase (Oka *et al.*, 1984) are normally present in eukaryotic cells and degrade this polymer. The antagonistic action of the synthetase on the one hand and glycohydrolase and lyase on the other are responsible for the very rapid turnover of poly(ADP-ribose) of 10 min or less (see, for example, Benjamin and Gill, 1980a). The venom phosphodiesterase has a special historical value since it was the first enzyme used to degrade poly(ADP-ribose) *in vitro* (Chambon *et al.*, 1966; Nishizuka *et al.*, 1967).

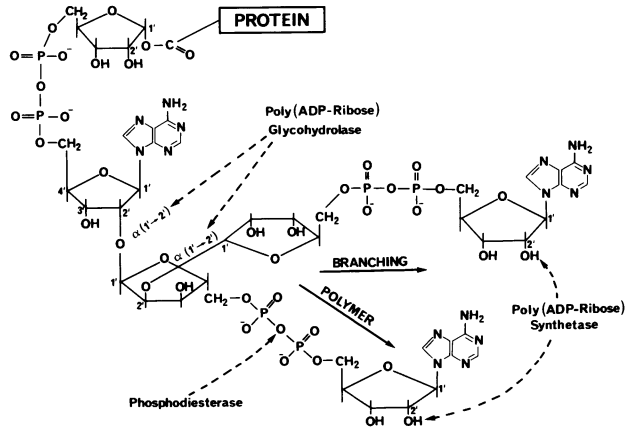

Fig. 1. The chemistry of poly(ADP-ribosylation) and enzymes involved in its synthesis and degradation. Some aspects of this modification are shown. The well-established attachment of ADP- ribose to protein carboxyl groups is depicted here, although additional types of linkage may exist. A branch of the polymer has been attached to the residue of ADP-ribose close to the protein, although branching occurs every ~30-50 residues (Miwa *et al.*, 1981). The arrow marked 'polymer' depicts the direction of the linear poly(ADP- ribose) backbone.

Several of the most frequently modified proteins are the enzyme poly(ADP-ribose) synthetase (Kawaichi *et al.*, 1981), histones (Ueda *et al.*, 1975; Burzio *et al.*, 1979; Ogata *et al.*, 1980a,b), high-mobility group (HMG) non-histone proteins (Reeves *et al.*, 1981), RNA polymerase I (Muller and Zahn, 1976), topoisomerase I (Ferro *et al.*, 1983; Jongstra-Bilen *et al.*, 1983), adenovirus T antigen and core proteins (Goding *et al.*, 1983), SV40 T antigen (Goldman *et al.*, 1981), heat-shock proteins (Carlsson and Lazarides, 1983), nucleolar proteins (Kawashima and Izawa, 1981) and so on.

A detailed analysis of the modified forms of histones is lacking. Furthermore, it is evident from published work that quantitative changes exist in the distribution of ADP-ribose groups among histones, poly(ADP-ribose) synthetase and other non-histones. These differences may be due to different

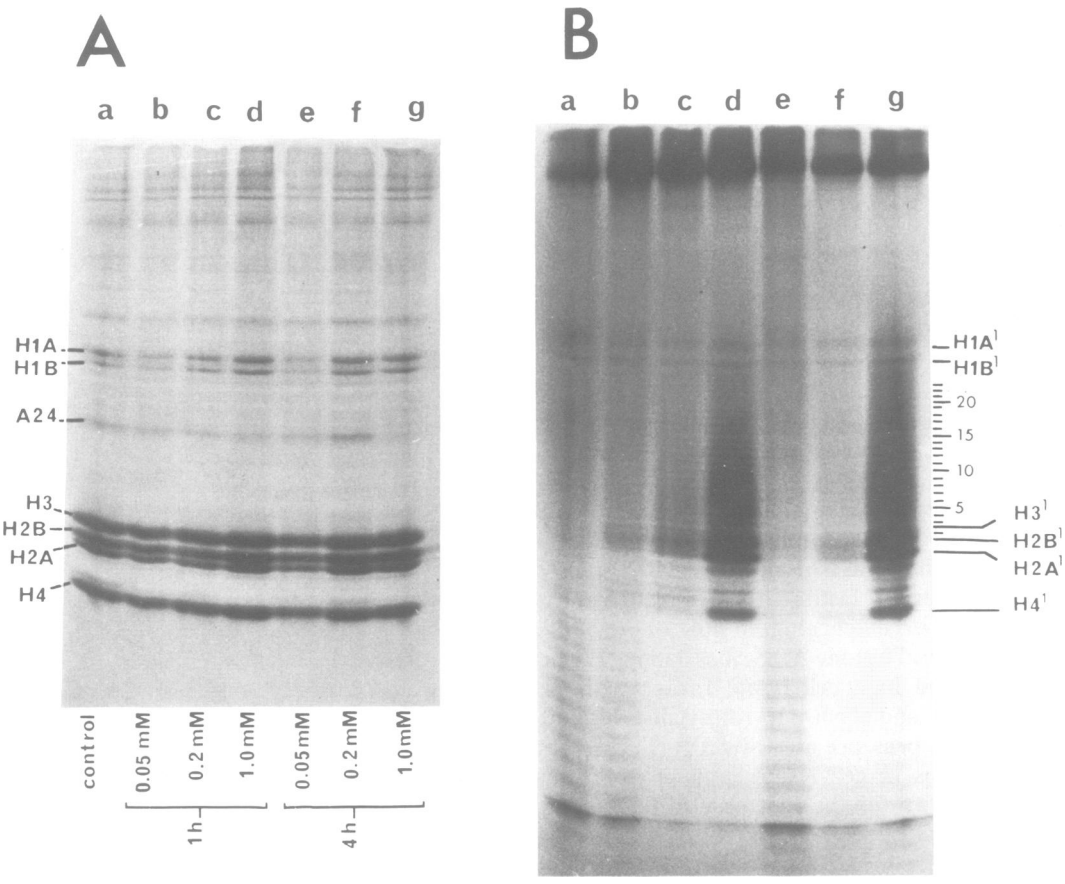

Fig. 2. Increase in histone ADP-ribosylation following treatment of mouse myeloma NS1 cells with DMS. Confluent cells were treated with DMS. Nuclei were isolated and incubated with radioactively labeled NAD^+ in the absence of cold NAD^+. Histones were analyzed on polyacrylamide gels in the presence of SDS (Laemmli, 1970). (**A**) Coomassie Blue staining of the SDS gel. (**B**) Autoradiogram of the same gel: **lane a**, control; **lanes b–d**, treatment of NS1 cells with increasing concentrations of DMS (as indicated at the bottom of Figure 2A) for 1 h; **lanes e–g**, treatment of NS1 cells with the same concentrations of DMS for 4 h. Histones are indicated to the left of A and to the right of B. The superscripts '1' to the histone symbols indicate the first band of each ADP-ribosylated histone, supposed to correspond to the mono(ADP-ribosylated) species. The numbers 5, 10, 15 and 20 represent the fifth, tenth, etc. band above histone H2B.

experimental protocols or cell types employed; alternatively they may be due to the rapid turnover of poly(ADP-ribose) (Benjamin and Gill, 1980a). For example, Okayama *et al.* (1978) have reported that 60% of the ADP-ribose groups are attached to basic proteins in rat liver nuclei using dihydroxyboryl chromatography. Among those proteins, histones H2B and H1 were found to contain 30% of the total ADP-ribose groups. Using also rat liver nuclei, Riquelme *et al.* (1979) have determined that 40% of the poly (ADP-ribose) was associated with basic proteins and about half of this was associated with histone H1. The same discrepancy holds for the ADP-ribose chain length associated with histones. A mean chain length of 2–3 was found for histone H1 isolated from Ehrlich ascites (Braeuer *et al.*, 1981) whereas Stone *et al.* (1977), Adamietz *et al.* (1978), Nolan *et al.* (1980) and Aubin *et al.* (1982) have identified 12–16 modification intermediates in ADP-ribosylated histone H1.

Although multiple histone H1 ADP-ribosylated intermediates have been identified up to a limiting form earlier thought to represent an H1–H1 dimer held by a chain of poly(ADP-ribose) but also identified as hyper(ADP-ribosylated) histone H1, i.e. histone H1 carrying ~15 ADP-ribose groups (De Murcia *et al.*, 1986), similar species for core histones have not been found.

We have undertaken a systematic study on histone ADP-ribosylation *in vitro* using two-dimensional gel electrophoresis. In this article we demonstrate that virtually all five histones can be found in poly(ADP-ribosylated) forms, containing in some cases at least 17 ADP-ribose groups each when histones in isolated nuclei are labeled. Parallel experiments in cell lysates also show the presence of multiple ADP-ribosylated forms of all histones. These conclusions are not modified when untreated cell cultures are employed. All previous studies have identified multiple ADP-ribosylated intermediates primarily for histone H1 and secondarily for H2B only. Thus, the theoretical number of variegated nucleosomes is significantly much higher than previously thought (Bafus *et al.*, 1978).

Results

Increase in histone ADP-ribosylation after DNA damage

An increase in histone poly(ADP-ribosylation) after treatment of confluent mouse myeloma cells in culture with the DNA-methylating agent dimethylsulfate (DMS) is shown in Figure 2. In this case, isolated nuclei were incubated under conditions favoring the incorporation of radioactive ADP-

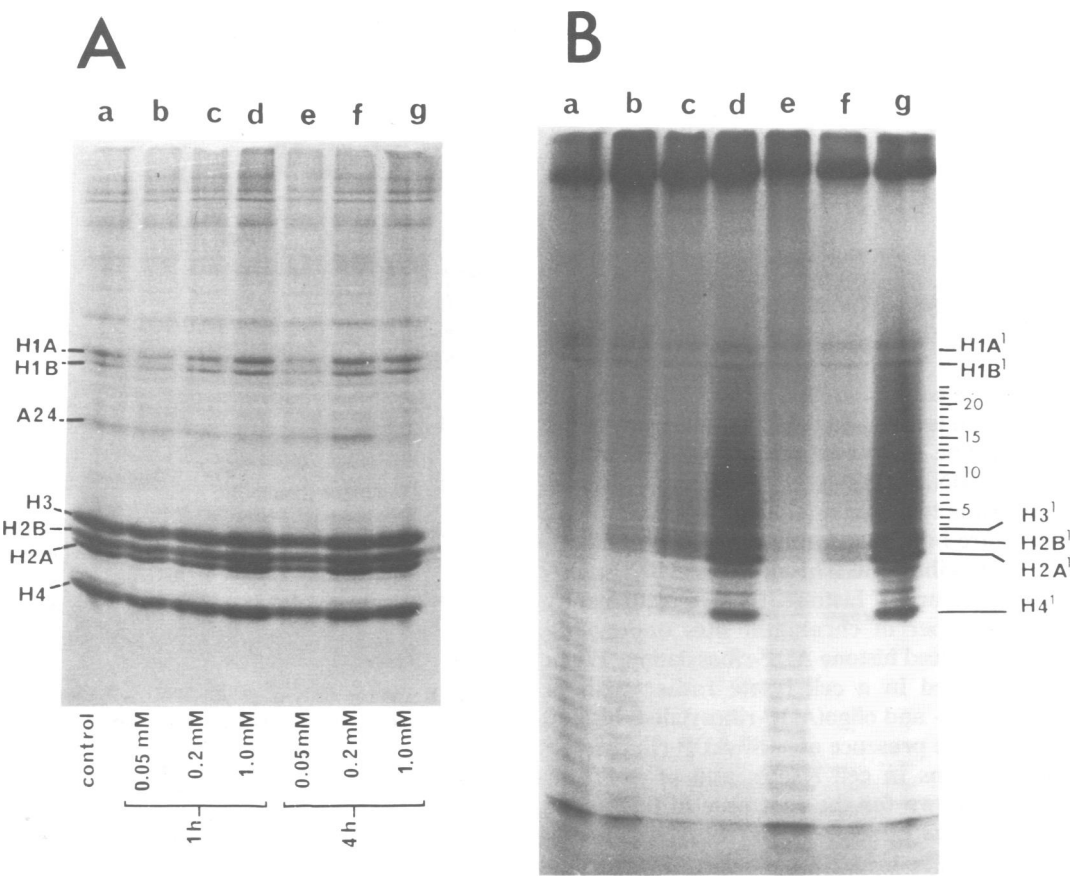

Fig. 2. Increase in histone ADP-ribosylation following treatment of mouse myeloma NS1 cells with DMS. Confluent cells were treated with DMS. Nuclei were isolated and incubated with radioactively labeled NAD^+ in the absence of cold NAD^+. Histones were analyzed on polyacrylamide gels in the presence of SDS (Laemmli, 1970). (**A**) Coomassie Blue staining of the SDS gel. (**B**) Autoradiogram of the same gel: **lane a**, control; **lanes b–d**, treatment of NS1 cells with increasing concentrations of DMS (as indicated at the bottom of Figure 2A) for 1 h; **lanes e–g**, treatment of NS1 cells with the same concentrations of DMS for 4 h. Histones are indicated to the left of A and to the right of B. The superscripts '1' to the histone symbols indicate the first band of each ADP-ribosylated histone, supposed to correspond to the mono(ADP-ribosylated) species. The numbers 5, 10, 15 and 20 represent the fifth, tenth, etc. band above histone H2B.

experimental protocols or cell types employed; alternatively they may be due to the rapid turnover of poly(ADP-ribose) (Benjamin and Gill, 1980a). For example, Okayama *et al.* (1978) have reported that 60% of the ADP-ribose groups are attached to basic proteins in rat liver nuclei using dihydroxyboryl chromatography. Among those proteins, histones H2B and H1 were found to contain 30% of the total ADP-ribose groups. Using also rat liver nuclei, Riquelme *et al.* (1979) have determined that 40% of the poly (ADP-ribose) was associated with basic proteins and about half of this was associated with histone H1. The same discrepancy holds for the ADP-ribose chain length associated with histones. A mean chain length of 2–3 was found for histone H1 isolated from Ehrlich ascites (Braeuer *et al.*, 1981) whereas Stone *et al.* (1977), Adamietz *et al.* (1978), Nolan *et al.* (1980) and Aubin *et al.* (1982) have identified 12–16 modification intermediates in ADP-ribosylated histone H1.

Although multiple histone H1 ADP-ribosylated intermediates have been identified up to a limiting form earlier thought to represent an H1–H1 dimer held by a chain of poly(ADP-ribose) but also identified as hyper(ADP-ribosylated) histone H1, i.e. histone H1 carrying ~15 ADP-ribose groups (De Murcia *et al.*, 1986), similar species for core histones have not been found.

We have undertaken a systematic study on histone ADP-ribosylation *in vitro* using two-dimensional gel electrophoresis. In this article we demonstrate that virtually all five histones can be found in poly(ADP-ribosylated) forms, containing in some cases at least 17 ADP-ribose groups each when histones in isolated nuclei are labeled. Parallel experiments in cell lysates also show the presence of multiple ADP-ribosylated forms of all histones. These conclusions are not modified when untreated cell cultures are employed. All previous studies have identified multiple ADP-ribosylated intermediates primarily for histone H1 and secondarily for H2B only. Thus, the theoretical number of variegated nucleosomes is significantly much higher than previously thought (Bafus *et al.*, 1978).

Results

Increase in histone ADP-ribosylation after DNA damage

An increase in histone poly(ADP-ribosylation) after treatment of confluent mouse myeloma cells in culture with the DNA-methylating agent dimethylsulfate (DMS) is shown in Figure 2. In this case, isolated nuclei were incubated under conditions favoring the incorporation of radioactive ADP-

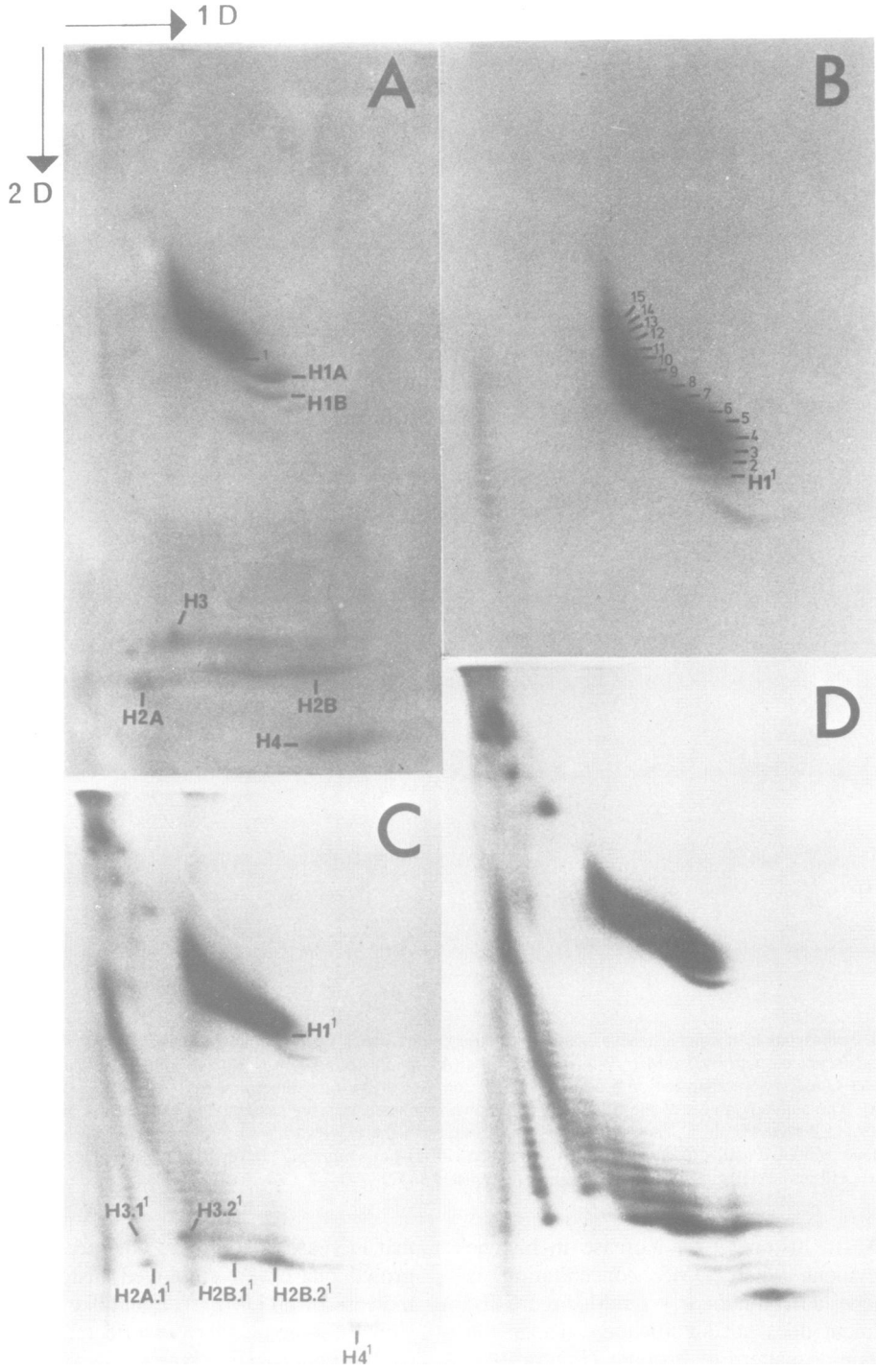

Fig. 4. Poly(ADP-ribosylated) forms of histone H1. Nuclei from NS1 cells in the exponential growth phase, continuously treated with 1 mM DMS for 3 h, were isolated and incubated as follows: **A** and **B**, 0.2 mM cold NAD^+ was added for 5 min and then 0.005 mM [^{32}P]NAD^+ was added for another 25 min; **C**, the same concentrations of cold and radioactive NAD^+ were simultaneously added for 30 min. **D**, 0.005 mM of radioactive NAD^+ was added for 5 min followed by chase with 0.2 mM of cold NAD^+ for 25 min. B is a higher magnification of the autoradiogram around ADP-ribosylated bands of H1 from A. In A, the autoradiogram and the Coomassie Blue-stained gel have been superposed and photographed together. There is no detectable labeling of core histones in A. Visible histone bands in A are Coomassie Blue-stained spots.

DMS for 1 and 4 h respectively. Treatment of cells with 2.5 mM DMS kills ~50% of the cells in 1 h, whereas at 12.5 mM for 1 h only ~10% of the cells survive. Incubation of cells for <30 min in DMS does not result in a noticeable increase in histone ADP-ribosylation under our labeling conditions (not shown).

Analysis of the ADP-ribosylation pattern of individual histones

The ADP-ribosylation pattern at different times of histones from NS1 cells continuously treated with 1 mM DMS for 2.5 h is shown in Figure 3. In this case nuclei were isolated and labeled; histones were extracted with acid−urea in the

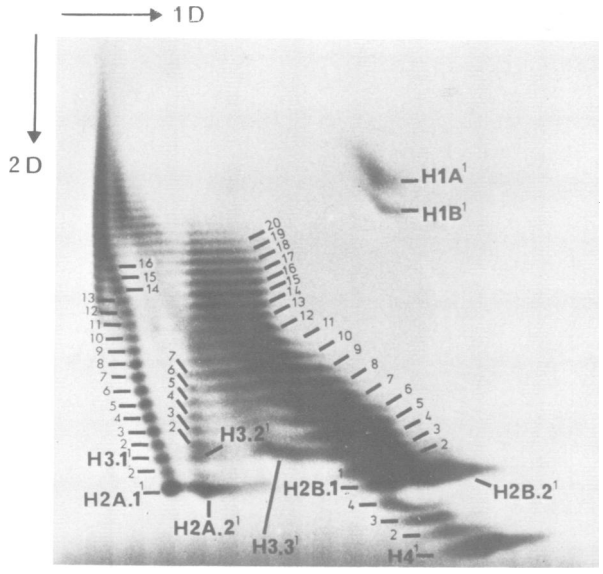

Fig. 5. Increase in core histone poly(ADP-ribosylation). Confluent mouse myeloma cells were continuously treated with 1 mM DMS for 1.5 h. Nuclei were isolated and incubated in the presence of 0.005 mM [^{32}P]NAD$^+$ and 0.2 mM cold NAD$^+$. Reactions were arrested after 8 min, nuclei were digested with micrococcal nuclease, lysed and histones analyzed on 6 M urea, 1% Triton gels and in the presence of SDS in the second dimension. The autoradiogram of the second-dimensional gel is shown. ADP-ribosylated species of each major variant histone are designated as in Figure 3.

presence of protamine to facilitate their displacement from DNA. Under these conditions DNA and most non-histones precipitate. Histones were then separated by PAGE in the presence of 6 M urea, 1% Triton, 1 M acetic acid and in a second dimension on an SDS gel. This type of analysis permits the identification of primary structure histone variants (Franklin and Zweidler, 1977; Boulikas, 1985b).

Labeling of histones in mouse nuclei with radioactive NAD$^+$ for 30 s using the endogenous poly(ADP-ribose) synthetase predominantly produces mono(ADP-ribosylated) histones when a low concentration of NAD$^+$ is employed (Figure 3A). The evidence that the fastest-migrating labeled histone spots on Figure 3A are mono(ADP-ribosylated) histones arises by superposing the autoradiogram and the Coomassie Blue stained and dried gel representing the non-modified histones. In this case, the mono-modified spots migrate slower than the stained spots and this retardation in mobility is consistent with the ladder of oligo(ADP-ribosylated) histones on the autoradiogram. At 30 s, 1 min, 4 min or 16 min of labeling (Figure 3A−D respectively) the mono(ADP-ribosylated) forms of histones predominate. However, due to a higher incorporation of radioactivity di-, tri-, etc., up to deca(ADP-ribosylated) forms can be visualized (Figure 3C,D).

Differences in the modification pattern among core histones can be seen. For example, histones H3 and H2B contain the highest concentration of oligo- compared with mono(ADP-ribosylated) species. Histone H4 seems to be present in up to tetra(ADP-ribosylated) forms (Figure 3C,D) although higher bands are present that are not detected due to their co-migration with the H2B ladder. Histone H2A.1 seems also to be present in mono- and di-modified forms although the presence of higher bands co-migrating with the H3.1 ladder cannot be excluded. Histone H1 is present in mono-modified forms.

Another interesting observation is that a histone H4 band, migrating slower than H4 on the first dimension supposed to represent H4-acetylated species (Figure 3C), is mainly present at tri- and tetra(ADP-ribosylated) species under conditions rendering the non-acetylated H4 mainly mono-(ADP-ribosylated). If this slower band of H4 (H4ac) indeed represents acetylated H4, then these data strengthen the notion that acetylated H4 is more frequently tri- and tetra-(ADP-ribosylated) and establish a link between the modifications. Provided that acetylated forms of H4 represent H4 intermediates in the assembly of newly made H4 with DNA (Louie and Dixon, 1972), then histone ADP-ribosylation may be implicated in histone assembly during DNA replication (T.Boulikas, in preparation).

In this and the previous experiment (Figures 2 and 3), histone H1 is mainly present as a mono(ADP-ribosylated) form and less heavily labeled than core histones. This does not necessarily mean that histone H1 is modified to a lesser extent than core histones. The subsequent experiment reveals that H1, like core histones, is also poly(ADP-ribosylated). Thus, the differences between the experiments shown in Figures 2 and 3 (as well as Figures 5 and 6, see below) on the one hand and the experiment shown in Figure 4 on the other may result from a much higher turnover rate of poly-(ADP-ribose) groups on H1 than on core histones.

Poly(ADP-ribosylation) of histone H1

In this experiment (Figure 4), cells from the exponential growth phase were employed, contrary to the experiments shown in Figures 2 and 3 (as well as Figures 5 and 6, see below) where confluent mouse cells were employed. Nuclei, isolated from these cells, were incubated with radioactively labeled NAD$^+$, (0.005 mM). Cold NAD$^+$ at a concentration of 0.2 mM was added 5 min before (Figure 4A,B), at the same time (C) or 5 min after (D) the addition of radioactive NAD$^+$s and poly(ADP-ribosylation) reactions were allowed to proceed for 30 min. A higher proportion of radioactive ADP-ribose groups are associated with histone H1 than with core histones, especially under the conditions of Figure 4A where almost all histone labeling occurs on H1.

When nuclei are pre-incubated for 5 min with cold NAD$^+$ prior to their labeling with [^{32}P]NAD$^+$, a preferential modification of histone H1 is observed (Figure 4A,B). At least 15 ADP-ribosylated species of histone H1 are present (marked in B) in agreement with previous studies (Stone *et al.*, 1977; Poirier *et al.*, 1982a,b). An increase in core histone modification relative to histone H1 is observed from A to C to D that probably reflects differences in the turnover rates of poly(ADP-ribose) associated with H1 or core histones. The overall incorporation of radioactivity increases ~1.5 times from A to C and about 2.2 times from A to D as was deduced by measuring the counts per minute in acid-extracted histone samples. However, the gels shown in Figure 4 have been selected from ~600 two-dimensional gels in a series of 61 independent experiments. Poly(ADP-ribosylated) forms of histone H1 as shown in Figure 4 were observed in ~5% of the gels and always in experiments employing exponentially growing cells. In most cases, however, H1 is mainly mono(ADP-ribosylated) as shown in Figures 2, 3, 5 and 6.

Again, in the experiment shown in Figure 4, multiple

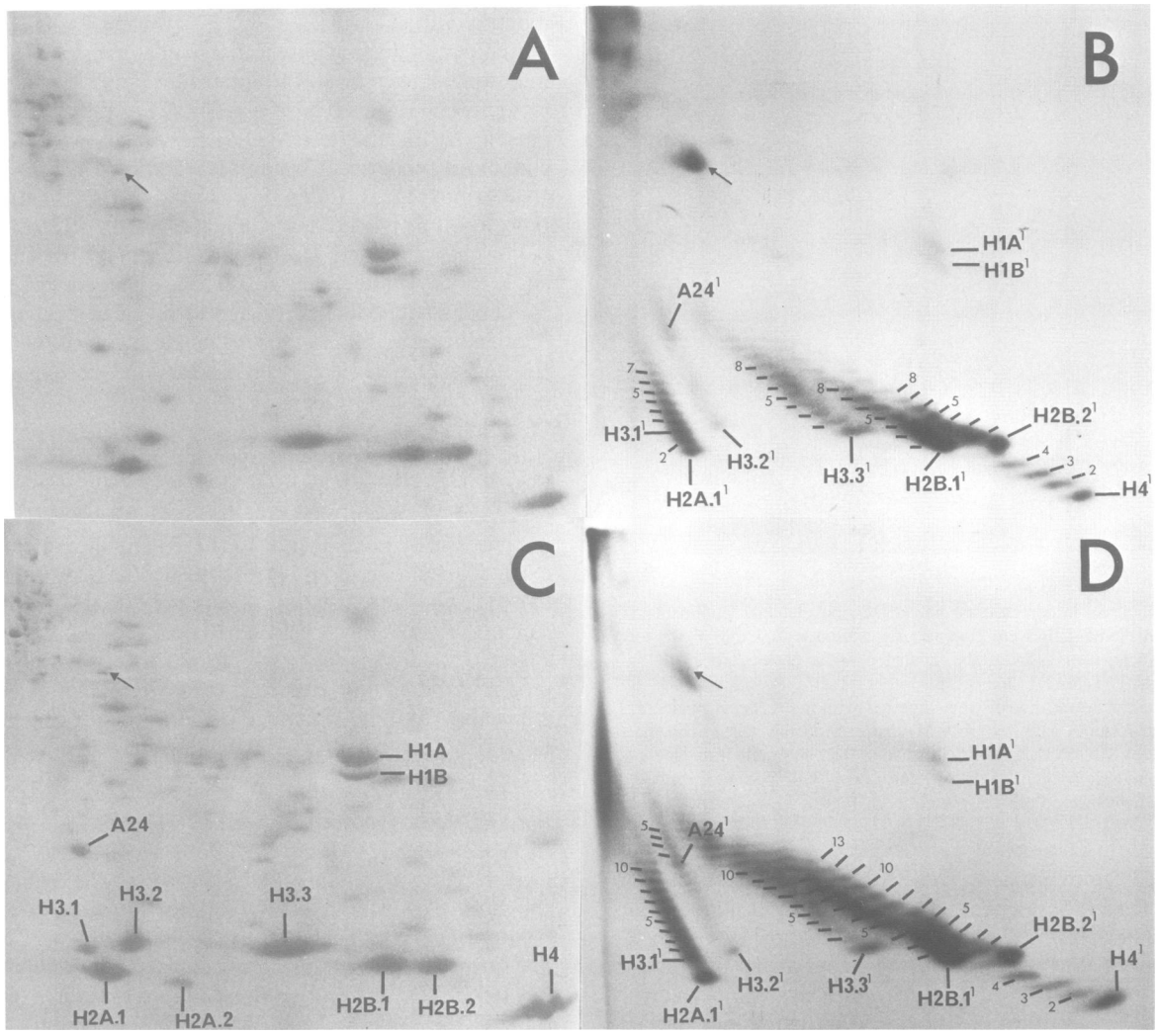

Fig. 6. Histone ADP-ribosylation in cell lysates and isolated nuclei from untreated cell cultures. Confluent mouse NS1 cells not treated with DMS were employed. Labeling was performed in cell lysates (**A,B**) in isolated nuclei (**C,D**) and histones were analyzed by two-dimensional gel electrophoresis. A and C show the Coomassie Blue staining of the gels and B and D the autoradiogram of the respective gels. The autoradiogram as shown in B was exposed three times longer than in D. The non-histone in B and D that is radiolabeled is marked with an arrow in A and C. The ADP-ribosylated histones are enumerated as in the legends to Figures 2 and 3.

bands are present on the H3 and H2B variants, in agreement with the data shown in Figure 3. Histone H4 displays up to four bands and H2A mostly two bands (Figure 4). Higher bands have been obtained for histone H4 on two-dimensional gels by increasing the Triton concentration in the first dimension (not shown). An increase in Triton concentration has been shown to retard the mobility of H4 on acetic acid–urea–Triton gels (Boulikas, 1985b). The H2A.2 variant has also been found in ~12 ADP-ribosylated forms on two-dimensional gels (Table I).

Increase in core histone poly(ADP-ribosylation)

When cold NAD^+ at a concentration of 0.2 mM was included in the reaction mixture, the ratio of poly- to mono-(ADP-ribosylated) core histones increased as shown in Figures 4 and 5 (cf. Figure 3). Furthermore, the employment of confluent cells (Figure 5) renders histone H1 mono-modified whereas employment of 0.2 mM NAD^+ results in a high degree of core histone poly(ADP-ribosylation). The presence of histone H1 in its mono(ADP-ribosylated) form is in agreement with Figures 2 and 3. The variant forms of H2B and H3 display at least 20 bands each, histone H4 at least four bands and H2A variants at least two bands (Figure 5).

Differences in ADP-ribosylated histones synthesized between cell lysates and isolated nuclei

Since isolated nuclei may be depleted in soluble components that are normally present in cells, an attempt was made to study histone ADP-ribosylation in cells and to compare this with that obtained in isolated nuclei. The drawback in such experiments is the impermeability of living cells to NAD^+; thus, the cell membrane needs to be modified in order to label the histones. For this purpose we have employed the non-ionic detergent Triton X-100 in order to dissolve the lipids in the membranes, and we performed the labeling of histones with radioactive NAD^+ in a very concentrated cell lysate (about 5–10 cell volumes). In parallel we have studied the effect of Triton concentration on histone ADP-ribosylation (T.Boulikas, in preparation). Our data show that a final

Table I. Approximate number of total ADP-ribosylated variant histones in mouse cells

Variant histone	No. of bands in each variant	
	In nuclei	In cell lysates
H1A	17	8
H1B	17	8
H2A.1	>2	>2
H2A.2	12	4
H2B.1	20	10
H2B.2	20	10
H3.1	20	8
H3.2	20	13
H3.3	20	12
H4	14	>4
Acetylated H4	>8	>8
Total no. of bands	170	87

concentration of 0.1% Triton employed in this study does not cause any significant changes in the pattern of histone ADP-ribosylation in isolated nuclei other than a 1.1-fold increase in the incorporation of radioactivity to histones.

Another argument may be that the pattern of multiple ADP-ribosylated variant histones is limited to repairing patches of chromatin since DMS-damaged cells were employed. The results shown in Figure 6 were obtained with untreated cell cultures; thus, the overall conclusion of this study on the presence of multiple ADP-ribosylated variant histones in DMS-treated cells also holds for non-damaged cultured cells.

Figure 6B reveals that the small fraction of histones that become labeled during incubation of cell lysates with radioactive NAD^+ seem to carry a smaller number of ADP-ribose groups than histones labeled in isolated nuclei (Figure 6D). For example, ~8 ADP-ribosylated histone forms are present in each of the H3.1, H3.3, H2B.1 and H2B.2 variants when labeling takes place in cell lysates (Figure 6B and Table I). This is in contrast to the presence of ~15 ADP-ribosylated forms for the same histone variants when labeling takes place in isolated nuclei (Figure 6D) or to ~20 forms detected in different experiments (Figure 5 and Table I). The Coomassie Blue staining pattern of the histones and non-histones does not display any differences when labeling is performed in cell lysates or isolated nuclei (compare A and C in Figure 6).

It is noteworthy that a great number of non-histones can be analyzed by our two-dimensional gel system. We can detect at least 70 non-histone bands in a typical gel (Figure 6A,C). To the best of our knowledge this is the first time that the analysis of such a great number of non-histones has been reported using a two-dimensional gel employing acetic acid–urea–Triton in the first dimension. We suppose that this is due to the high concentration of urea employed in the preparation of our samples during histone extraction from whole nuclei (see Materials and methods).

Another striking difference when the ADP-ribosylation of histones is performed in cell lysates versus isolated nuclei is the total amount of radioactivity incorporated into the histones under identical labeling conditions (not shown). Among seven independent experiments the incorporation of radioactivity to either histones (acid–urea-soluble proteins) or to total 25% trichloroacetic acid-precipitable material is 1.5–6 times higher when isolated nuclei rather than cells are employed. In the particular experiment shown in Figure 6, a 5-fold difference in the histone labeling was found. In most experiments mono(ADP-ribosylated) histones are the main constituents in the labeled ladder on each histone in cell lysates, compared with the poly(ADP-ribosylation) pattern obtained in isolated nuclei.

Total number of ADP-ribosylated variant histones

The overall conclusion of this study on the presence of multiple ADP-ribosylated forms of histones is thus confirmed, employing cell lysates in addition to isolated nuclei (Figure 6) as well as cells that were rendered permeable to NAD^+ by a hypotonic shock (T.Boulikas, in preparation). The approximate numbers of ADP-ribosylated forms for each variant histone observed in cell lysates or isolated nuclei are summarized in Table I. These numbers are approximate; they are based on the number of bands in the ADP-ribosylation ladder of variant histones on the two-dimensional gels shown in Figures 3–6 as well as on the great number of two-dimensional gels obtained in our laboratory.

The total number of H1 forms is an underestimate due to the presence of H1 microheterogeneity. The two main H1A and H1B bands can be further resolved into two to three species each that, along with the $H1_0$, increase the number of H1 species to about six (Boulikas, 1987a). Notwithstanding the possibility of underestimation of the H1 bands, we have measured ~170 ADP-ribosylated species of all variant histones in nuclei compared with ~87 such species in cell lysates (Table I). However, the most important question that remains to be answered is to what extent histone–histone contacts are disrupted in nucleosomes containing poly(ADP-ribosylated) histones and the upper limit number of ADP-ribose groups a particulate histone–histone interaction can sustain.

Discussion

Histone ADP-ribosylation after DNA damage

An increase in poly(ADP-ribose) synthetase activity following damage of cells with DNA-methylating agents (Smulson *et al.*, 1977; Juarez-Salinas *et al.*, 1979; Durkacz *et al.*, 1980), nucleases (Berger *et al.*, 1978; Halldorsson *et al.*, 1978; Benjamin and Gill, 1980b; Cohen *et al.*, 1982), UV light (Jacobson *et al.*, 1983) or X-rays (Benjamin and Gill, 1980a) has been shown. This effect is inhibited by nicotinamide or 3-aminobenzamide, both potent inhibitors of poly(ADP-ribose) synthetase (James and Lehmann, 1982; Sims *et al.*, 1982), and is accompanied by a dramatic decrease in the cellular pool of NAD^+ (Smulson *et al.*, 1977; Jacobson *et al.*, 1980). NAD^+ is consumed to synthesize large amounts of poly(ADP-ribose) that can entrain cell death. Nicotinamide or 3-aminobenzamide retard the lowering of NAD^+ in cells treated with DNA-methylating agents (Sims *et al.*, 1982).

The studies addressing the increase in poly(ADP-ribose) synthetase activity were mainly based on the incorporation of radioactivity from NAD^+ to trichloroacetic acid-precipitable material (for example, see Berger *et al.*, 1979; Durkacz *et al.*, 1980; Benjamin and Gill, 1980b; Sims *et al.*, 1982; Jacobson *et al.*, 1983; Morgan and Cleaver, 1983). Protein acceptors in DMS or otherwise treated and untreated cells have not been determined except in some cases. Adamietz

and Rudolph (1984), for example, have found that more than half of the nuclear mono- and poly(ADP-ribosyl) residues were linked to histone H2B after DMS treatment of hepatoma cells. In this case aminophenyl−boronic acid−agarose chromatography was employed to isolate the ADP-ribosylated from the non-modified histones. However, mainly mono-modified histones were found in this case.

Stone *et al.* (1977) have identified a complex pattern of 15−16 bands of ADP-ribosylated histone H1 in HeLa nuclei. A similar complex of H1 was also observed by Adamietz *et al.* (1978) and Nolan *et al.* (1980) that was believed to represent a dimer of H1 cross-linked by a 15-unit chain of poly(ADP-ribose). Aubin *et al.* (1982) have identified histone H1 as the main poly(ADP-ribosylated) histone with 12 modification intermediates in rat pancreas. This poly(ADP-ribosylated) species of H1 resembled the 'H1 dimer' and induced chromatin relaxation (Poirier *et al.*, 1982a,b; Aubin *et al.*, 1983). An analysis of the histone acceptors of ADP-ribose has also been attempted by Levy-Wilson (1983) in *Tetrahymena* using two-dimensional gel electrophoresis, but mono-modified core histones were found in this case. Analysis of ADP-ribosylated histones by two-dimensional gel electrophoresis has also been employed by Huletsky *et al.* (1985) and Gaudreau *et al.* (1986); in this case mainly hyper(ADP-ribosylated) forms of histone H1 and secondarily of histone H2B were found.

To our knowledge, the presence of at least 15 ADP-ribosylated species for each variant core histone is clearly demonstrated for the first time in this work. We have also studied histone ADP-ribosylation in untreated cells (Figure 6); these data show similar patterns in histone ADP-ribosylation and, thus, the conclusions that can be drawn from the work presented in this paper are not uniquely due to DMS treatment of cells.

Type of linkage between histones and poly(ADP-ribose)

The data in Figure 2B suggest that a decrease in the amount of free poly(ADP-ribose) takes place during DMS treatment of mouse myeloma cells. Two main enzymes, poly(ADP-ribose) glycohydrolase (Miwa *et al.*, 1974) and ADP-ribosyl protein lyase (Oka *et al.*, 1984) have been recognized for the degradation of protein-associated poly(ADP-ribose) *in vivo*. Since the first enzyme is an exoglycosidase (Miwa *et al.*, 1974) and the second enzyme acts only upon mono-(ADP-ribosylated) proteins (Oka *et al.*, 1984) the free poly(ADP-ribose) bands below H4 in Figure 2B must arise by chemical hydrolysis of the histone−poly(ADP-ribose) linkage.

Why the linkage between poly(ADP-ribose) and histones or other nuclear proteins becomes less labile in cells treated with DMS is not clear. Adamietz *et al.* (1978) have identified two types of linkage between histones and poly(ADP-ribose). One of them is sensitive to hydrolysis by hydroxylamine at neutral pH and most probably involves carboxyl histone groups. The other type of linkage is more resistant to hydrolysis, requiring alkaline conditions, and probably employs amino groups of histones. It may be that DMS favors histone modification via the second type of linkage, this being more resistant to chemical hydrolysis.

Resolution of this problem requires enzymatic or specific chemical cleavage of the histones and identification of the peptides within the known primary structures of the histones carrying this modification (Boulikas *et al.*, 1980).

Extent of histone H1 and core histone ADP-ribosylation

Our results indicate that histone H1 is present as a mono-(ADP-ribosylated) species when nuclei from confluent cultured cells are employed (Figures 2, 3 and 5) as opposed to core histones which can also exist in poly(ADP-ribosylated) forms. However, employment of nuclei from cells in the exponential growth phase has resulted in the identification of poly(ADP-ribosylated) forms of H1 (Figure 4). The extent of histone ADP-ribosylation in our experiments greatly depends on the concentration of NAD^+, on whether cells from the confluent or exponential growth phase are employed, on whether cell lysates or isolated nuclei are labeled, on whether the DNA has or has not been digested prior to labeling as well as on other variations in the labeling mixture. At low concentration of NAD^+, core histones are mainly present as mono(ADP-ribosylated) forms (Figure 3), whereas at 0.2 mM NAD^+, assimilating the physiological nuclear concentration of NAD^+ (Rechsteiner *et al.*, 1976) core histones appear as mono-, oligo- and poly(ADP-ribosylated) species (Figures 4−6).

Adamietz *et al.* (1978) have detected mono-modified histone H1 in intact, proliferating HeLa cells, as opposed to poly(ADP-ribosylated) H1 synthesized by isolated HeLa nuclei. Our results showing that the formation of poly- or mono-modified histones in isolated nuclei depends on the experimental conditions is in agreement with the data of Adamietz *et al.* (1978) and Huletsky *et al.* (1985).

In a number of studies showing the presence of hyper-(ADP-ribosylated) histone H1, exogenous poly(ADP-ribose) synthetase was added in order to enhance the efficiency of the incorporation of radioactivity from NAD^+ to histones. For example, De Murcia *et al.* (1986) and Poirier *et al.* (1982a) have employed poly(ADP-ribose) synthetase from calf thymus for the ADP-ribosylation of histones in polynucleosomes. In this case virtually 100% of the H1 was present in its hyper(ADP-ribosylated) form, most probably corresponding to the upper limit ADP-ribosylated band of H1 in Figure 4B in this study, as well as to the 'H1 dimer' band in the work published by Stone *et al.* (1977), Butt and Smulson (1980), Nolan *et al.* (1980) and by others. It is, thus, possible that exogeneously added poly(ADP-ribose) synthetase preferentially modifies histone H1 to a high extent.

Mono-, oligo- and poly-modified forms of H1 and core histones must be hierarchically produced in the nucleus and serve a specific function. We think that they participate in chromatin replication (T.Boulikas, in preparation) other than their demonstrated function in chromatin repair (Ueda and Hayaishi, 1985; Boulikas, 1987a). Their role may be exerted by the modification of histone−histone interactions in higher-order chromatin structures, in nucleosomes or in histone complexes assembled during chromatin replication. Poly (ADP-ribosylated) species of H1 may also modify higher-order chromatin structures (Poirier *et al.*, 1982a) or H1−core histone contacts at the chromatosome level (Thoma *et al.*, 1979; Boulikas *et al.*, 1980; Boulikas, 1985a,b).

Our data demonstrate that multiple bands first observed for ADP-ribosylated H1 are also present in core histones. The regular spacing of core histone oligo(ADP-ribosylated)

bands during their analysis on two-dimensional gels (Figures 2–6) suggests that each one differs from its neighbors by one ADP-ribose group. It is curious that core histones (Figures 5, 6D) do not also display upper-limit ADP-ribosylated bands as does histone H1 (De Murcia *et al.*, 1986).

That each band on the poly(ADP-ribosylation) ladder of H3 and H2B variants differs from its neighbors by one ADP-ribose group can also be deduced from the data shown in Figure 5. About 22 bands of H2B supposed to represent H2B with 1–22 groups of ADP-ribose display a diminishing electrophoretic mobility starting at H2B and terminating near H1B on SDS gels (Figure 5). Considering that the mol. wt of one ADP-ribose group is ~500, that of H2B 15 000, then that of the docosane (ADP-ribosylated) H2B is 26 000, roughly coinciding with the apparent mol. wt of H1 on SDS gels. The actual mol. wt of H1 is ~22 000, but from SDS gels an apparent mol. wt of 26 000–28 000 is deduced due to its high content in lysine (Boulikas, 1987a).

The high accessibility of H2B and H3 histones to poly(ADP-ribose) synthetase and the low build up of poly(ADP-ribose) groups on histones H1, H2A and H4 (Figures 5 and 6) may have important implications in chromatin structure that are not clearly understood. We very often observe significant changes in the ADP-ribosylation pattern among variants of the same histone: for example H3.1, H3.2 and H3.3 in Figure 6; such changes may reflect the different role variant forms may play in chromatin structure.

The results in Figures 3 and 4 suggest that acetylated forms of H4 are more easily tri- and tetra(ADP-ribosylated). We may thus speculate that both modifications occurring in a concerted fashion are important intermediates in the deposition of newly made H4 on the replication fork (T.Boulikas, in preparation).

Sites for ADP-ribosylation on histones H4, H3 and H2A have not yet been determined. The glutamic acid residue at amino acid position 2 of H2B that is ADP-ribosylated (Burzio *et al.*, 1979), although absent in sea urchins and *Drosophila*, has been evolutionarily conserved in mammals, frogs, fish, etc. (Von Holt *et al.*, 1979). The ADP-ribosylation of H2B is confined to the N-terminal region where most modifications occur (Boulikas, 1987a). Glutamic acid residues on histone H4—the most evolutionarily conserved protein known today, after ubiquitin—are present at amino acid positions 52, 53, 63 and 74 (De Lange *et al.*, 1969) and are, thus, not confined to the N-terminal end. However, we cannot exclude the possibility that ADP-ribosylation of H4 occurs on amino acid residues other than glutamic acid.

Nucleosome complexity due to variant and modified histones

The idea of the epigenetic information stored in the form of histone–DNA interactions that needs to be maintained and accurately propagated during chromatin replication to daughter cells has been entertained by Tsanev and Sendov (1971), as well as by Weintraub *et al.* (1978). Relevant to this issue is the question of nucleosome phasing in which histone octamers seem to occupy unique positions with respect to DNA sequences, at least in several genes (reviewed by Boulikas, 1987a). Non-histones may participate in epigenetic mechanisms by excluding nucleosome formation on DNA sequences specifically recognized and occupied by such proteins. However, nucleosome reconstitution experiments from histones and DNA excluding the presence of non-histones show that histone octamers are precisely placed on cloned DNA (Chao *et al.*, 1979), suggesting their sequence preference. DNA sequence preference of octamers may change with compositionally different octamers, although this question has not yet been experimentally tested. Fifty-four possible histone octamers have been calculated with unique composition in variant histones; this number is increased by several orders of magnitude considering the acetylated, phosphorylated and methylated forms of histones (Bafus *et al.*, 1978). The addition of at least 60 ADP-ribosylated histone species in this study to the known post-translational histone complexity greatly enhances the number of theoretically possible variegated nucleosomes. Their actual contribution to nucleosome functional complexity *in vivo* needs to be investigated.

Materials and methods

Cell culture

Mouse myeloma NS1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 10% fetal calf serum (Flow), 2 mM glutamine (Sigma), 0.0025 mg/ml fungizone, 50 IU/ml penicillin and 0.005 mg/ml streptomycin (Flow). Cells were cultured in 250 ml polystyrene tissue culture flasks (Falcon).

Treatment of cells with DMS

A solution of 250 mM of DMS (Aldrich Chemical Co.) was freshly prepared in DMEM medium as a stock and was then added at concentrations up to 5 mM directly to cultured cells. Cells were continuously treated with DMS by incubation at 37°C for 30 min–4 h.

Nuclei isolation

Cells from ~50 ml of cell culture (~2.5×10^7 cells total) were collected by centrifugation at 800 *g* for 5 min using 50-ml plastic tubes (Corning) and a small table centrifuge. The cell pellet was drained and resuspended in 0.5 ml of 0.3 M sucrose, 50 mM Tris–HCl, pH 6.8, 25 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.01% 2-mercaptoethanol (nuclei buffer) (Boulikas, 1985a). Cells were lysed and membranes were removed by the addition of 0.05 ml 10% Triton X-100 (Sigma) followed by the addition of 0.55 ml of 80% glycerol in nuclei buffer minus sucrose. The nuclei suspension was well mixed in an Eppendorf tube, 0.2 ml of 60% glycerol in nuclei buffer minus sucrose was underlayed and nuclei were purified by centrifugation through the cushion of glycerol for 4 min in a microfuge (~10 000 *g*). The supernatant was removed and nuclei were resuspended in ~0.7 ml of nuclei buffer.

ADP-ribosylation reactions in isolated nuclei

To the final nuclei preparation, stock solutions of dithiothreitol and $MgCl_2$ were added to 10 mM final concentrations, followed by the addition of 7.5×10^{-7} mmol (0.025 mCi) [adenylate-^{32}P]NAD^+ (New England Nuclear) and cold NAD^+ (Boehringer Mannheim) to 0.2 mM. The final reaction volume was 0.1 ml. This corresponds to 0.0075 mM in radioactive NAD^+ The order of addition of cold and radioactive NAD^+ was important. In some experiments the addition of cold NAD^+ was omitted. Nuclear ADP-ribosylation reactions employing the endogenous poly(ADP-ribose) synthetase were allowed to proceed for 30 min at 20°C at pH 6.8. Enzymatic reactions were quenched by the addition of nicotinamide solution (Aldrich) to 10 mM and incubation at 20°C for 5 min. Nuclei were pelleted in a microfuge for 40 s using Eppendorf tubes resuspended in 0.01 ml of nuclei buffer containing 1 U micrococcal nuclease (Worthington) and digested for 5 min at 20°C to reduce viscosity during sample preparation.

ADP-ribosylation of histones in cell lysates

The cell pellet (~2.5×10^7 cells) obtained as described above was resuspended into 0.05 ml of nuclei buffer followed by the addition of 0.05 ml of 0.4 mM cold NAD^+, 0.025 mCi [^{32}P]NAD^+, 20 mM $MgCl_2$, 20 mM dithiothreitol, 0.2% Triton X-100 and the ADP-ribosylation was allowed to proceed for 30 min at 20°C at pH 6.8. In this case, nuclei isolated from the same batch of cells within 5 min were incubated under identical conditions in the presence of 0.1% Triton (data shown in Figure 6). ADP-

ribosylations in the cell lysate were quenched by the addition of 0.45 ml nuclei buffer containing 0.1% Triton and 10 mM nicotinamide. Nuclei isolation and nuclease digestion were performed as described above.

Sample preparation and gel electrophoresis

To the nuclei suspension after nuclease digestion containing radioactively labeled ADP-ribose groups on histones, 0.1 ml 2 mM EDTA, 3 mM Tris−HCl, pH 6.8 were added. To an aliquot of the lysed nuclei an equal volume of 2% SDS, 8 M urea, 0.1% 2-mercaptoethanol, 0.01% bromophenol blue (SDS sample cocktail) was added for electrophoresis on polyacrylamide gels containing SDS (Laemmli, 1970). Electrophoresis on polyacrylamide gels containing 1 M acetic acid, 6 M urea and 0.25% (w/v) Triton X-100 was according to the method of Bonner *et al.* (1980) and Boulikas (1985b). To the lysed nuclei an equal volume of 0.4 M HCl, 5 mg/ml protamine sulfate (Sigma), 8 M urea and 0.01% Pyronin Y (Bio-Rad) was added (acid−urea sample cocktail) causing the solubilization of histones and several non-histones and the precipitation of DNA and most non-histones. The clear supernatant, after precipitation of the DNA by centrifugation for 2 min in Eppendorf tubes, was loaded on the acidic gels.

Second-dimensional gel electrophoresis

The first-dimensional 0.5-mm-thick acetic acid−urea−Triton gels were fixed, stained and destained over a period of 2−3 h as described (Boulikas, 1985b). They were then equilibrated in water for 5 h with two or three changes, and finally incubated under mild shaking for 30 min in 2% SDS, 0.1% 2-mercaptoethanol, 60 mM Tris−HCl, pH 6.8, 10% glycerol. Strips were cut and immediately layed on top of a 0.75-mm-thick gel (Laemmli, 1970).

Acknowledgements

I thank Sylvie Berardi for artwork and technical assistance and Madeleine Paquin for typing the manuscript. This work was supported by grant A 3372 from the Natural Sciences and Engineering Research Council of Canada and a grant from the Centre de Recherche Clinique de l'Université de Sherbrooke. The author is chercheur-boursier du Fonds de la Recherche en Santé du Québec.

References

Adamietz,P. and Rudolph,A. (1984) *J. Biol. Chem.*, **259**, 6841−6846.
Adamietz,P., Bredehorst,R. and Hilz,H. (1978) *Eur. J. Biochem.*, **91**, 317−326.
Alvarez-Gonzales,R. and Jacobson,M.K. (1985) In Althaus,F.R., Hilz,H. and Shall,S. (eds), *ADP-Ribosylation of Proteins.* Springer-Verlag, Berlin, pp. 32−36.
Aubin,R.J., Dam,V.T., Miclette,J., Brousseau,Y., Huletsky,A. and Poirier, G.G. (1982) *Can. J. Biochem.*, **60**, 1085−1094.
Aubin,R.J., Fréchette,A., De Murcia,G., Mandel,P., Lord,A., Grondin,G. and Poirier,G.G. (1983) *EMBO J.*, **2**, 1685−1693.
Bafus,N.L., Albright,S.C. Todd,R.D. and Garrard,W.T. (1978) *J. Biol. Chem.*, **253**, 2568−2574.
Benjamin,R.C. and Gill,D.M. (1980a) *J. Biol. Chem.*, **255**, 10493−10501.
Benjamin,R.C. and Gill,D.M. (1980b) *J. Biol. Chem.*, **255**, 10502−10508.
Berger,N.A., Sikorski,G.W., Petzold,S.J. and Kurohara,K.K. (1979) *J. Clin. Invest.*, **63**, 1164−1171.
Berger,N.A., Weber,G. and Kaichi,A.S. (1978) *Biochim. Biophys. Acta*, **519**, 87−104.
Bonner,W.M., West,M.H.P. and Stedman,J.D. (1980) *Eur. J. Biochem.*, **109**, 17−23.
Boulikas,T. (1985a) *Can. J. Biochem. Cell Biol.*, **3**, 1022−1032.
Boulikas,T. (1985b) *Anal. Biochem.*, **149**, 379−386.
Boulikas,T. (1987a) *Int. Rev. Cytol. Suppl.*, **17**, 493−571.
Boulikas,T. (1987b) *Int. Rev. Cytol. Suppl.*, **17**, 599−684.
Boulikas,T., Wiseman,J.M. and Garrard,W.T. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 127−131.
Braeuer,H.C., Adamietz,P., Nellessen,U. and Hilz,H. (1981) *Eur. J. Biochem.*, **114**, 63−68.
Burzio,L.O., Riquelme,P.T. and Koide,S.S. (1979) *J. Biol. Chem.*, **254**, 3029−3037.
Butt,T.R. and Smulson,M. (1980) *Biochemistry*, **19**, 5235−5242.
Carlsson,L. and Lazarides,E. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4664−4668.
Chambon,P., Weill,J.D., Doly,J., Strosser,M.T. and Mandel,P. (1966) *Biochem. Biophys. Res. Commun.*, **25**, 638−643.
Chao,M.V., Gralla,J. and Martinson,H.G. (1979) *Biochemistry*, **18**, 1068−1074.
Cohen,J.J., Catino,D.M., Petzold,S.J. and Berger,N.A. (1982) *Biochemistry*, **21**, 4931−4940.
De Lange,R.J., Fambrough,D.M., Smith,E.L. and Bonner,J. (1969) *J. Biol. Chem.*, **244**, 5669−5679.
De Murcia,G., Huletsky,A., Lamarre,D., Gaudreau,A., Pouyet,J., Daune, M. and Poirier,G.G. (1986) *J. Biol. Chem.*, **261**, 7011−7017.
Durkacz,B.W., Omidiji,O., Gray,D.A. and Shall,S. (1980) *Nature*, **283**, 593−596.
Ferro,A.M., Higgins,N.P. and Olivera,B.M. (1983) *J. Biol. Chem.*, **258**, 6000−6003.
Franklin,S.G. and Zweidler,A. (1977) *Nature*, **266**, 273−275.
Gaudreau,A., Ménard,L., De Murcia,G. and Poirier,G.G. (1986) *Biochem. Cell Biol.*, **64**, 146−153.
Goding,C.R., Shaw,C.H., Blair,G.E. and Russell,W.C. (1983) *J. Gen. Virol.*, **64**, 477−483.
Goldman,N., Brown,M. and Khoury,G. (1981) *Cell*, **24**, 567−572.
Halldorsson,H., Gray,D.A. and Shall,S. (1978) *FEBS Lett.*, **85**, 349−352.
Huletsky,A., Niedergang,C., Fréchette,A., Aubin,R., Gaudreau,A. and Poirier,G.G. (1985) *Eur. J. Biochem.*, **146**, 277−285.
Jacobson,E.L., Antol,K.M., Juarez-Salinas,H. and Jacobson,M.K. (1983) *J. Biol. Chem.*, **258**, 103−107.
Jacobson,M.K., Levi,V., Juarez-Salinas,H., Barton,R.A. and Jacobson,E.L. (1980) *Cancer Res.*, **40**, 1797−1802.
James,M.R. and Lehmann,A.R. (1982) *Biochemistry*, **21**, 4007−4013.
Jongstra-Bilen,J., Ittel,M.E., Niedergang,C., Vosberg,H.-P. and Mandel,P. (1983) *Eur. J. Biochem.*, **136**, 391−396.
Juarez-Salinas,H., Sims,J.L. and Jacobson,M.K. (1979) *Nature*, **282**, 740−741.
Kawaichi,M., Ueda,K. and Hayaishi,O. (1980) *J. Biol. Chem.*, **255**, 816−819.
Kawaichi,M., Ueda,K. and Hayaishi,O. (1981) *J. Biol. Chem.*, **256**, 9483−9489.
Kawashima,K. and Izawa,M. (1981) *J. Biochem.*, **89**, 1889−1901.
Laemmli,U.K. (1980) *Nature*, **227**, 680−685.
Levy-Wilson,B. (1983) *Biochemistry*, **22**, 484−489.
Louie,A.J. and Dixon,G.H. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1975−1979.
Miwa,M., Tanaka,M., Matsushima,T. and Sugimura,T. (1974) *J. Biol. Chem.*, **249**, 3475−3482.
Miwa,M., Ishihara,M., Takishima,S., Takasuka,N. and Maeda,M. (1981) *J. Biol. Chem.*, **256**, 2916−2921.
Morgan,W.F. and Cleaver,J.E. (1983) *Cancer Res.*, **43**, 3104−3107.
Muller,W.E.G. and Zahn,R.K. (1976) *Mol. Cell. Biochem.*, **12**, 147−159.
Nishizuka,Y., Ueda,K., Nakazano,K. and Hayaishi,O. (1967) *J. Biol. Chem.*, **242**, 3164−3171.
Nolan,N.L., Butt,T.R., Wong,M., Lambrianidou,A. and Smulson,M.E. (1980) *Eur. J. Biochem.*, **113**, 15−25.
Ogata,N., Ueda,K. and Hayaishi,O. (1980a) *J. Biol. Chem.*, **255**, 7610−7615.
Ogata,N., Ueda,K., Kagamiyama,H. and Hayaishi,O. (1980b) *J. Biol. Chem.*, **255**, 7616−7620.
Oka,J., Ueda,K., Hayaishi,O., Komura,H. and Nakanishi,K. (1984) *J. Biol. Chem.*, **259**, 986−995.
Okayama,H., Ueda,K. and Hayaishi,O. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1111−1115.
Poirier,G.G., de Murcia,G., Jongstra-Bilen,J., Niedergang,C. and Mandel, P. (1982a) *Proc. Natl. Acad. Sci. USA*, **79**, 3423−3427.
Poirier,G.G., Niedergang,C., Champagne,M., Mazen,A. and Mandel,P. (1982b) *Eur. J. Biochem.*, **127**, 437−442.
Rechsteiner,M., Hillyard,D. and Olivera,B.M. (1976) *Nature*, **259**, 695−696.
Reeves,R., Chang,D. and Chung,S.-C. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6704−6708.
Riquelme,P.T., Burzio,L.O. and Koide,S.S. (1979) *J. Biol. Chem.*, **254**, 3018−3028.
Sims,J.L., Sikorski,G.W., Catino,D.M., Berger,S.J. and Berger,N.A. (1982) *Biochemistry*, **21**, 1813−1821.
Smulson,M.E., Schein,P., Mullins,D.W., Jr and Sudhakar,S. (1977) *Cancer Res.*, **37**, 3006−3012.
Stone,P.R., Lorimer,W.S., III and Kidwell,W.R. (1977) *Eur. J. Biochem.*, **81**, 9−18.
Thoma,F., Koller,T. and Klug,A. (1979) *J. Cell Biol.*, **83**, 403−427.
Tsanev,R. and Sendov,B. (1971) *J. Theor. Biol.*, **30**, 337−393.

Ueda,K. and Hayaishi,O. (1985) *Annu. Rev. Biochem.*, **54**, 73–100.
Ueda,K., Narumiya,S., Miyakawa,N. and Hayaishi,O. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 516–523.
Ueda,K., Omachi,A., Kawaichi,M. and Hayaishi,O. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 205–209.
Ueda,K., Kawaichi,M., Okayama,M. and Hayaishi,O. (1979) *J. Biol. Chem.*, **254**, 679–687.
Von Holt,C., Strickland,W.N., Brandt,W.F. and Strickland,M.S. (1979) *FEBS Lett.*, **100**, 201–218.
Weintraub,H., Flint,S.J., Leffak,I.M., Groudine,M. and Grainger,R.M. (1978) *Cold Spring Harbor Symp. Quant. Biol.*, **42**, 401–407.

Received on August 11, 1987; revised on October 22, 1987