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DNA strand breaks alter histone ADP-ribosylation

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ABSTRACT **Histone ADP-ribosylation was studied using two-dimensional gel electrophoresis after cleavage of the nuclear DNA with nucleases. Modified histones carrying different numbers of ADP-ribose groups form a ladder of bands above each variant histone. Cellular lysates containing unfragmented DNA mainly synthesize mono(ADP-ribosylated) histones. Cleavage of the DNA with either DNase I or micrococcal nuclease to fragments of an average size of 10–20 kilobases (kb) dramatically induces the formation of poly(ADP-ribosylated) species of histones in nuclei. As the number of DNA strand breaks produced by either DNase I or micrococcal nuclease increases and a great number of DNA cuts is introduced (fragments of 0.4–0.2 kb), the size of the poly(ADP-ribose) chains on the histones decreases. Finally, in the presence of 10 mM cAMP as an inhibitor of poly(ADP-ribose) glycohydrolase, human lymphoid nuclei synthesize hyper(ADP-ribosylated) histone H2B with at least 40 ADP-ribose groups attached to it. Lateral ladders emanating at precise points of the linear ladder on hypermodified H2B can arise from branching of poly(ADP-ribose) or from multiple monomodifications of glutamic (or aspartic) acid residues. Branching or *de novo* monomodifications occur after a precise number of ADP-ribose groups have been added to a histone molecule. Poly(ADP-ribosylated) histones thus appear to be intermediates in nuclear processes involving DNA strand breaks.**

The composition and structure of a particular chromatin region are responsible for the transcriptional activity of the DNA sequences associated with this region. Although proteins displaying DNA sequence specificity play an important role in the differential gene expression, histone modifications including acetylation, phosphorylation, ADP-ribosylation, ubiquitination, and methylation also participate in major nuclear functions (reviewed in refs. 1 and 2). Histone modifications require specific enzymes for their addition and removal (3) added in a highly ordered manner with respect to the particular histone site (4). Furthermore, histone modifications display alterations during development (5), the cell cycle (6), the particular chromatin fraction within the same cell type (7), or the variant form within the same histone fraction (8).

Two distinct types of enzymes are responsible for the transfer of adenosine diphosphate ribose (ADP-ribose) groups from NAD^+ to proteins: mono-ADP-ribosyltransferases catalyze the transfer of only one ADP-ribose to arginine, lysine, glutamic acid, or diphthamide residues of proteins (9, 10). The second type of enzyme, poly(ADP-ribose) synthetase, localized in the nucleus, catalyzes both the mono(ADP-ribosylation) of proteins and the subsequent addition of a second, third, *n*th such group to the modified site (reviewed in ref. 10).

Poly(ADP-ribosylation) is the most dramatic postranslational modification of histones and a discrete number of other nuclear proteins (reviewed in refs. 10–12). This modification is probably implicated in all nuclear processes involving DNA strand breaks like replication (e.g., ref. 13), repair (e.g., ref. 14), or recombination (15). In addition, poly(ADP-ribosylation) is involved in RNA synthesis (16), cellular differentiation (e.g., ref. 17), aging (18), cell growth (19), heat shock response (20), or nuclear matrix structure (21).

Poly(ADP-ribose) isolated from eukaryotic cells exhibits variable chain length ranging from 1 to 200 residues and shows a branched structure (22). Poly(ADP-ribose) synthetase depends for its activity on DNA and appears to protect 60–90 base pairs (bp) of DNA from nuclease digestion, although longer stretches of DNA seem to be wrapped about the enzyme molecule (23).

A significant increase in the activity of poly(ADP-ribose) synthetase takes place concomitant with the appearance of DNA strand breaks produced by nucleases (24). Restriction fragments with flush ends are 3–10 times more efficient than restriction fragments with 5′ protruding or recessed ends in stimulating the activity of this enzyme (25). DNA restriction fragments with dephosphorylated rather than phosphorylated termini are more efficient activators of ADP-ribosylation (26). Closed circular plasmid DNAs are ineffective for synthetase activation unless linearized with endonucleases (25).

Previous studies have revealed the presence of a ladder of 1–20 ADP-ribosylated species on each major variant histone (27). It was of interest to learn the pattern of ADP-ribosylation in histones found in nucleosomes containing internal or linker DNA strand breaks. Both single-stranded and double-stranded DNA breaks activate poly(ADP-ribose) synthetase. Such breaks are produced by nucleases, topoisomerases, etc., and participate in important nuclear functions such as in DNA repair or replication, transcription, or changing the torsional strain in a particular chromatin loop (reviewed in ref. 2). Since it is difficult to obtain native nucleosome preparations containing the low level of endogenous DNA strand breaks [only 100–300 single strand breaks per diploid genome have been calculated (28) in differentiated myoblasts], massive damage of the DNA in nuclei has been induced with nucleases and histone ADP-ribosylation has been examined. Since DNase I attacks both linker and nucleosome DNA and gives DNA fragments mainly containing 5′ recessed ends as opposed to micrococcal nuclease that attacks linker DNA and gives fragments with 5′ protruding ends (29), the pattern of histone ADP-ribosylation has been compared after digestion of nuclei with either enzyme as well as with that obtained in nuclei containing unfragmented DNA. The data show a dramatic dependence of histone ADP-ribosylation upon the extent of DNA cuts produced by either nuclease in isolated nuclei.

MATERIALS AND METHODS

Mouse mastocytoma P815 and human chronic myelogenous leukemia K-562 cells (obtained from American Type Culture Collection) were cultured in RPMI medium supplemented with 10% fetal bovine serum, 20 units of penicillin G per ml, and 0.2 mg of streptomycin sulfate per ml (GIBCO). Nuclei were isolated through a glycerol cushion in Eppendorf tubes as described (27). Histone poly(ADP-ribosylation) in isolated

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nuclei or in cell lysates as well as sample preparation and two-dimensional gel electrophoresis were performed as described (27).

Two-dimensional gels were dried, and sets of four were placed in 35.5 × 43 cm^2 cassettes and exposed to Kodak X-Omat film at −70°C for several days using an intensifying screen (DuPont Cronex, Lightning Plus). Dried gels and their corresponding autoradiographs were photographed at the same distance using a 35-mm camera.

RESULTS

Histone ADP-Ribosylation in the Presence or Absence of DNA Strand Breaks. Dramatic differences in the pattern of histone ADP-ribosylation during histone labeling with radioactive NAD^+ was obtained in cell lysates containing unfragmented DNA (Fig. 1 *A* and *B*) compared to their labeling in nuclease-digested nuclei (Fig. 1 *C* and *D*). Histones were labeled in cell lysates or in isolated nuclei and analyzed by two-dimensional gel electrophoresis (27). Putative mono-(ADP-ribosylated) variant histones are mainly produced in the cell lysate (Fig. 1*B*). Higher bands are also present but in very low concentration, presumably representing di-, tri-, etc., ADP-ribosylated species of variant histones.

This is in contrast with the presence of an ADP-ribosylation ladder of variant histones when their labeling is performed in micrococcal nuclease-digested nuclei (Fig. 1*D*). Each band in the ladder of the modified histone species is generally regularly spaced from its adjacent bands and is believed to represent a certain size class of a poly(ADP-ribosylated) histone differing from its adjacent species by one ADP-ribose group. Thus, in the absence of DNA strand breaks histones are mainly mono(ADP-ribosylated) but oligo- and poly(ADP-ribosylated) histone species are produced after micrococcal nuclease cleavage of the DNA. Since the ADP-ribosylation ladder on histones is not visible upon Coomassie blue staining (Fig. 1 *A* and *C*), modified histones represent a small fraction of total histones.

Histone ADP-Ribosylation in Extensively Nuclease-Cleaved Chromatin. Fig. 2*A* shows that nuclei containing mildly DNase I-cleaved DNA (20 kb average size of DNA fragments) maintain some of their total histones as ADP-ribosylated species, each containing 1–29 ADP-ribose groups. When chromatin is reduced into mono- and oligonucleosomes with micrococcal nuclease (average 0.4-kb fragments) the length of poly(ADP-ribose) on histones is considerably diminished (Fig. 2*B*). Histones are mainly present as mono- to hepta(ADP-ribosylated) forms; histones with 8–25 ADP-ribose groups are present in very low concentration. In this instance the concentration of a certain poly(ADP-ribosylated) histone form considerably decreases as the number of ADP-ribose groups associated with this molecule increases.

At least six ADP-ribosylated species of histone H4 are seen in Fig. 2 *A* and *B*. Higher bands may be masked by the H3.2 and H3.3 ADP-ribosylation ladder. When DNA is digested to an average of 20-kb fragments with DNase I, an increase in the concentration from di- to hexa(ADP-ribosylated) H4 is observed (Fig. 2*A*). This is in contrast to the decrease in the concentration of the bands of H4 in the order hexa- to

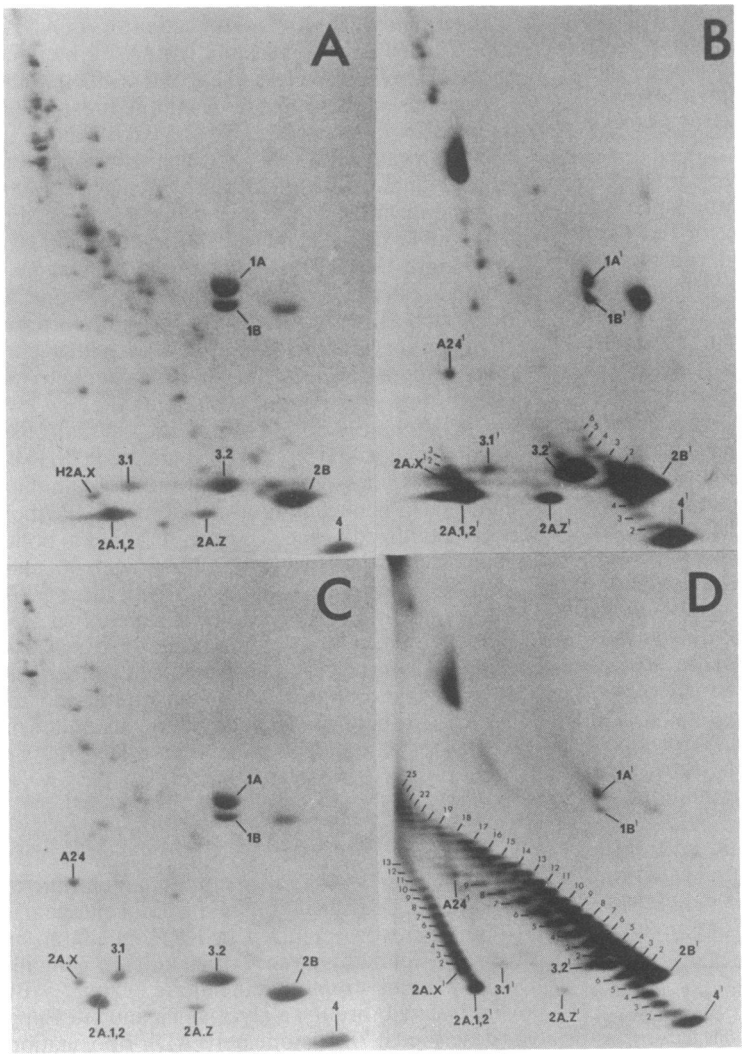

FIG. 1. Mono- or poly(ADP-ribosylated) histones are synthesized in the absence or presence of DNA strand breaks, respectively. Undigested cell lysates or micrococcal nuclease-digested mouse nuclei [2.5 kilobases (kb) average DNA size] whose two-dimensional protein patterns are shown in *A* or *C*, respectively, were incubated with [^{32}P]NAD^+. (*B* and *D*) Poly(ADP-ribosylated) variant histones purified from undigested cell lysates or micrococcal nuclease-digested nuclei, respectively. Variant histone forms are labeled in *A* and *C*; the letter H in the histone symbol is omitted for simplicity. The superscript "1" to the histone symbol in *B* and *D* represents the putative mono-(ADP-ribosylated) species; numbers 2–25 represent the putative di- to pentacosa(ADP-ribosylated) species of variant core histones. Identical exposure times during autoradiography were employed in *B* and *D*. The ADP-ribosylated species form a diagonal line above the position where the unmodified variant histone migrates.

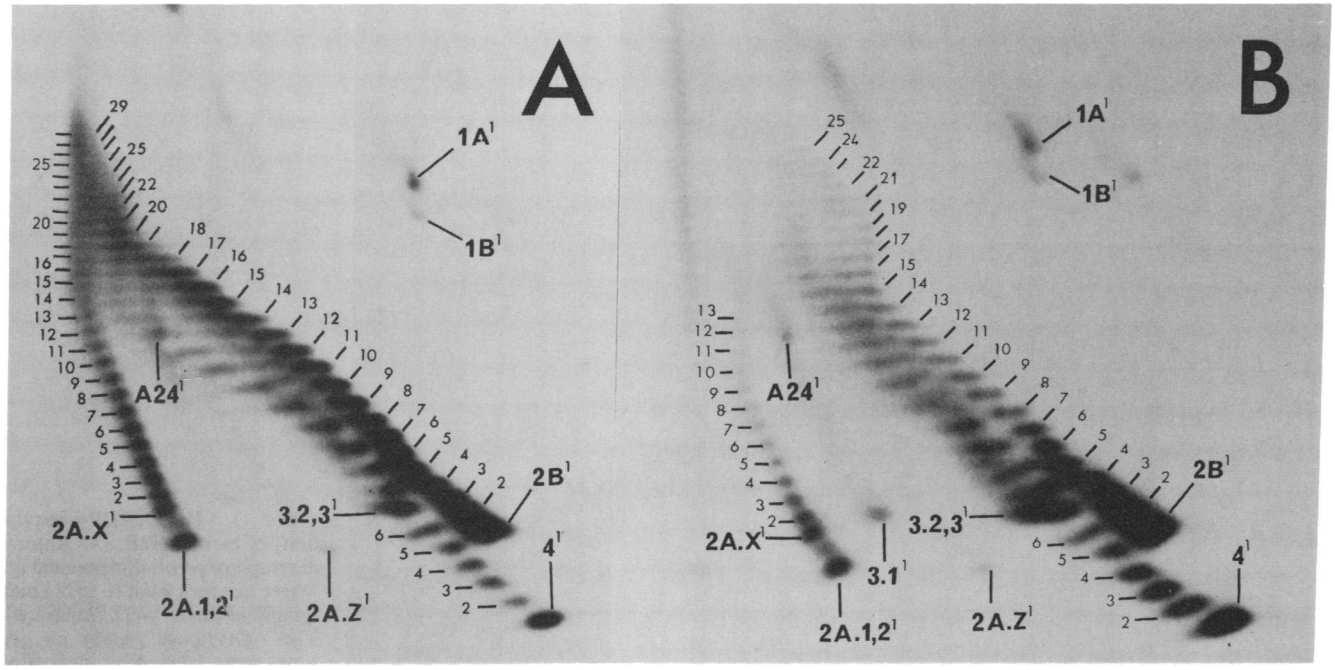

FIG. 2. Decrease in the length of poly(ADP-ribose) attached to histones in extensively micrococcal nuclease-digested nuclei. Autoradiographs of two-dimensional gels of histones ADP-ribosylated in mouse nuclei after digestion with DNase I to fragments of an average size of 20 kb (*A*) or digested with micrococcal nuclease to fragments of an average size of 0.4 kb (*B*). Identical amounts of histones were loaded and the dried gels were exposed for an identical amount of time during autoradiography.

di(ADP-ribosylated) species in nuclei following extensive digestion of chromatin to mononucleosomes (Fig. 2*B*).

Structure of Poly(ADP-Ribose) Attached to Mouse and Human Histone H2B. One additional difference in the ADP-ribosylation pattern of histones between Fig. 2 *A* and *B* is the point where the slope of the ADP-ribosylation ladder on histone H2B changes. This occurs beyond the 20th band in Fig. 2*A* but beyond residue 15 in Fig. 2*B*. Extensive studies in my laboratory show that such differences are not due to the use of micrococcal nuclease or DNase I but rather to the extent of digestion of mouse nuclei.

Two interpretations for the change in the slope of the poly(ADP-ribosylation) ladder are offered. (*i*) It may be due to the monomodification of one or more new sites on histone H2B, at glutamic (or aspartic) acid residues. (*ii*) It may be due to branching of the poly(ADP-ribose) occurring at the 15th (Fig. 2*B*) or 20th (Fig. 2*A*) residue of ADP-ribose away from the H2B molecule.

Appearance of lateral bands at multiple points on the ADP-ribosylation ladder has been observed during incubation of DNase I-digested nuclei obtained from K-562 human cultured cells with radioactive NAD^+. The labeling was performed in the presence of 10 mM cAMP as an inhibitor of poly(ADP-ribose) glycohydrolase (30). The autoradiograph of the two-dimensional gel of histones extracted from these nuclei is shown in Fig. 3*A*. The first side chain appears beyond the 16th band on the modification ladder on histone H2B. Therefore the bands of the first lateral ladder falling almost on a vertical line are marked 16.1, 16.2, 16.3, etc. (Fig. 3*A*). A second side chain occurs at the 17th residue and a third occurs at the 18th residue of the ladder in Fig. 3*A*.

Fig. 3*B* shows an independent experiment performed under conditions identical to those shown in Fig. 3*A*. In this case, the lateral ladders emanating from the 16th and 17th residues have come closer together compared to Fig. 3*A*. This is not surprising given the complexity of the molecules analyzed. The similarity in the general characteristics in the overall shape and pathway of the ladders on each variant histone on the autoradiographs of the two-dimensional gels is astonishing between the two experiments (Fig. 3 *A* and *B*).

One additional feature observed in Fig. 3 *A* and *B* is that the lateral chain that is built at the 18th ADP-ribose residue on H2B displays in turn an additional stagger and slope change around its 10th residue. The bands in this lateral ladder are marked 18.10.1, 18.10.2, etc., in Fig. 3*A*.

The presence of two or three bands on the same horizontal line of the autoradiograph of the two-dimensional gel at points of ladder staggering (Fig. 3 *A* and *B*) may be due to H2B acetylation or phosphorylation. Such modifications retard the mobility of histones on acidic but not on SDS gels and could justify the presence of spots to the left of the main spot.

DISCUSSION

DNA Strand Breaks and Histone ADP-Ribosylation. In the absence of DNA strand breaks histones are mono(ADP-ribosylated); however, when DNA strand breaks are produced, histones appear as poly(ADP-ribosylated) species (Fig. 1). The accumulation of mono(ADP-ribosylated) histones (Fig. 1*B*) may be due to the reduced activity of poly(ADP-ribose) synthetase (24, 25). In this case, the endogenous poly(ADP-ribose) glycohydrolase (30) will degrade the polymer on histones. Extensive studies have shown that poly(ADP-ribose) is rapidly synthesized and degraded in the nucleus with a half-life as low as 30 sec (31). ADP-ribosyl protein lyase (32) is the rate-limiting step in the overall turnover of poly(ADP-ribose) residues on histones. This explanation is supported by the studies of Wielckens *et al.* (31), who have observed a retarded removal of the primary ADP-ribose group after treatment of Ehrlich ascites cells with a DNA-degrading agent.

DNA strand breaks are responsible for activating poly(ADP-ribose) synthetase (e.g., refs. 24 and 25). According to my experiments, introduction of DNA strand breaks seems to induce the poly(ADP-ribosylation) of histones and not only the builtup of polymer on poly(ADP-ribose) synthetase as suggested from several studies (e.g., ref. 33).

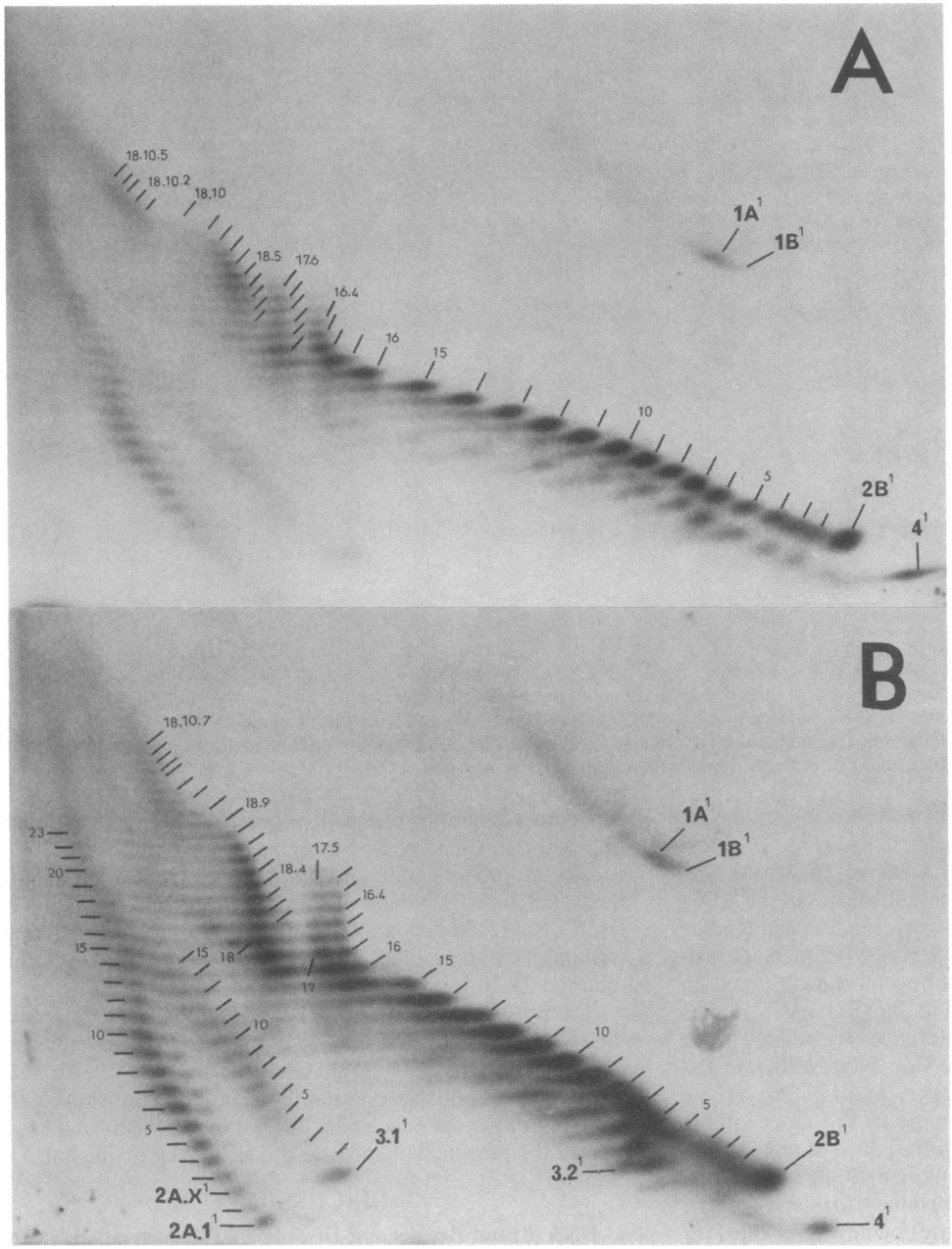

FIG. 3. Hyper(ADP-ribosylation) of human H2B. (*A*) Autoradiograph of a two-dimensional gel where histones from K-562 human lymphoid cells were separated. The ADP-ribose groups on histones were labeled by incubation of DNase I-digested nuclei to average 2.5 kb with [^{32}P]NAD$^+$. Histone labeling was performed in the presence of 10 mM cAMP in order to inhibit poly(ADP-ribose) glycohydrolase (30). (*B*) Results of an independent experiment. Bands on the modification ladder of H2B are enumerated from 1 to 18. The three side ladders emanating upward at bands 16, 17, and 18 are depicted as 16.1–16.6, 17.1–17.6, and 18.1–18.10 and most probably represent branched chains of poly(ADP-ribose) or chains growing at new sites on H2B (see text and models in Fig. 4). The H2B modification bands marked 18.10.1–18.10.5 represent intermediates of an additional chain of poly(ADP-ribose) either attached to a different amino acid residue on H2B or growing as a branch on preexisting chains of poly(ADP-ribose).

Poly(ADP-ribose) groups of much greater length may be attached to the histones but cannot be detected because they are not able to enter the first-dimensional acidic gel of polyacrylamide. Indeed, a number of studies suggest that poly(ADP-ribose) ranging in size from 1 to 200 residues may be synthesized in permeable tissues notwithstanding the limitations of the methods and the question of whether or not such data reflect the *in vivo* situation (reviewed in ref. 12). It can be estimated that at pH 3.5 about 26–31 ADP-ribose groups are required to completely neutralize the net positive charge of each core histone (or about 70 ADP-ribose groups for histone H1). This presumably represents the theoretical maximal number of ADP-ribosylated bands on each core histone that can enter the acid/urea/Triton X-100 gel. It is surprising that Fig. 3 displays more than about 35 bands on the modification ladder of human H2B entering the acid/urea/Triton X-100 gel.

Structure of Poly(ADP-Ribosylated) Histone H2B. The regularity of ADP-ribosylation ladders attached to histone variants is interrupted at precise points by band staggering or by a slope change. This change in the slope greatly depends on the extent of nuclease digestion but not on the use of either micrococcal nuclease or DNase I. One plausible explanation accounting for the change in the slope of the poly(ADP-ribosylation) ladder is that additional glutamic or aspartic acid residues on histones become mono(ADP-ribosylated) (Fig. 4*A*). The loss of their negative charge is accounted for by the gain of a negative charge from adenosine diphosphoribose. Thus, the overall charge of the histone molecule is not considerably altered; for example, the H2B species 18.1–18.4 in Fig. 3 migrate with almost identical mobilities in the first dimensional acid/urea/Triton X-100 gel.

One alternative explanation for the observed changes in the slope of the poly (ADP-ribosylation) ladder on histones at defined points is that they are due to polymer branching (Fig. 4*B*). ADP-ribose residues added at a branch in this case will not significantly alter the mobility of the modified histone on the acid/urea/Triton X-100 first-dimensional gel, but they will do so in the presence of SDS in the second dimension. This will be in analogy to the anomaly in the migration of curved DNA fragments on polyacrylamide gels (34). Branching of poly(ADP-ribose) either in its free form or attached to poly(ADP-ribose) synthetase has been demonstrated by chemical methods (35) as well as by electron microscopy (22).

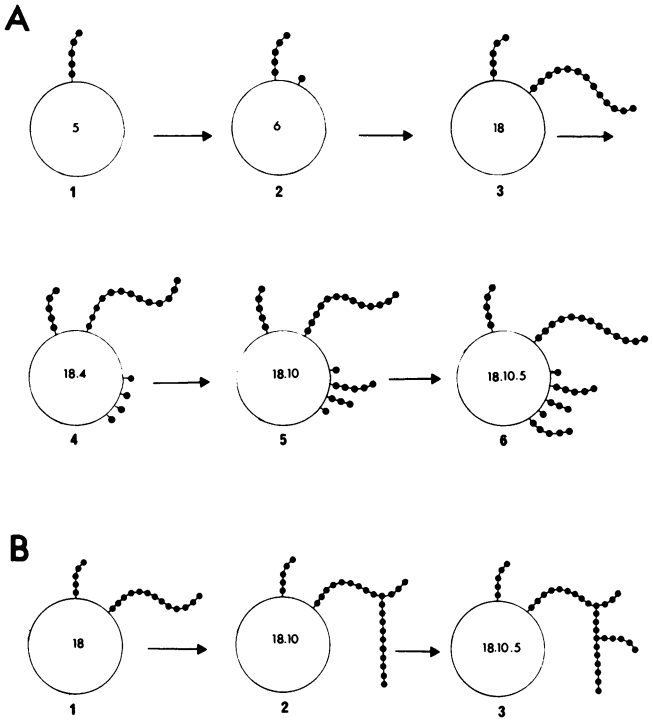

FIG. 4. Two models explaining the steps leading to the enzymatic hyper(ADP-ribosylation) of human histone H2B. In the multiple *de novo* monomodification model in *A*, human histone H2B is monomodified and a short chain of 5 residues of ADP-ribose is built on this site (step 1). This is suggested from the stagger between bands 5 and 6 on the H2B ladder (e.g., Fig. 3*B*). One additional monomodification takes place (step 2) and a poly(ADP-ribose) chain grows on the second site, giving a total of 18 ADP-ribose residues on both sites (step 3). Four new monomodifications take place, adding a total of 22 residues on histone H2B (step 4). This accounts for the abrupt slope change of the bands 18.1–18.4 in Fig. 3. The data shown in Fig. 3 are consistent with the addition of 6 more ADP-ribose groups in any combination to the preexisting mono- or poly-modified sites, leading to octacosa(ADP-ribosylated) H2B (step 5). At least 5 more residues of ADP-ribose are added to a new site, accounting for bands 18.10.1–18.10.5 in Fig. 3, leading to hyper(ADP-ribosylated) H2B with at least 33 residues of ADP-ribose (step 6). Although individual bands on the H2B modification ladder beyond this point cannot be distinguished, the ladder seems to continue. At least 10 ADP-ribose residues seem to be further added to histone H2B, giving a total of at least 43 ADP-ribose residues (Fig. 3). According to the model shown in *B*, poly(ADP-ribose) on H2B has a branched structure. The octadeca(ADP-ribosylated) H2B in step 1 is modified with a branch of 10 residues of ADP-ribose (step 2). This branch has been attached to the 10th residue of ADP-ribose on the previous chain although other possibilities exist. Finally, a new branch of 5 residues is added to the previous chain (step 3).

The data suggest that poly(ADP-ribose) attached to histones may have a defined branched structure (Figs. 1–3).

According to the second model (Fig. 4*B*), the mobility of poly(ADP-ribosylated) histones during electrophoresis on the acid/urea/Triton X-100 gel in the first dimension decreases more by the addition of one ADP-ribose group to the distal end of the polymer resulting in a linear chain than by the addition of a new ADP-ribose group at an internal residue of the polymer giving a branched structure.

The conditions that lead to the synthesis of histones modified with short or long chains of poly(ADP-ribose) have been established. These data are useful in order to explore using crosslinking the effect of mono-, oligo-, poly-, or hyper(ADP-ribosylation) of histones on histone–histone or histone–DNA interactions. This information will help our understanding of the role that this modification may play in chromatin functions by transiently remodeling chromatin structure.

A dramatic increase in the length of poly(ADP-ribose) on histones upon introduction of DNA cuts by nucleases has also been shown. Since DNA strand breaks are produced during DNA repair, replication, recombination, chromatin remodeling during development, or changing of DNA superhelicity by topoisomerases (reviewed in ref. 2), then poly(ADP-ribosylated) histones must constitute important intermediates in these nuclear processes.

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