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Decreased Heterogeneity of CS Histone Variants After Hydrolysis of the ADP-Ribose Moiety

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Abstract Sea urchin CS histone variants are electrophoretically heterogeneous when analyzed in two dimensional polyacrylamide gels (2D-PAGE). Previous results suggested that this heterogeneity is due to the poly (ADP-ribosylation) of these proteins. Consequently, native CS histone variants were subjected to different treatments to remove the ADP-ribose moiety. The incubation in 1 M hydroxylamine was not effective in eliminating the polymers of ADP-ribose from CS variants, and the treatment with sodium hydroxide was deleterious to the proteins. In contrast, the ADP-ribose moiety was successfully removed from the CS variants by incubation with phosphodiesterase (PDE). To eliminate contamination of CS histone variants with PDE extract, the enzyme was covalently bound to Sepharose 4B prior to its utilization. Treatment of native CS histone variants with this immobilized phosphodiesterase removed around 85% of the total ADP-ribose moiety from these proteins. After S-PDE treatment the complex electrophoretic pattern of CS histone variants in 2-D PAGE decreases to five major fractions. From these results we conclude that the electrophoretic heterogeneity of native CS histone variants is mainly due to the extent to which five main CS histone variants are poly(ADP)-ribosylated).

Key words: histone variants, poly(ADP-ribosylation), chromosomal proteins, sea urchin, development

In sea urchins the expression of distinct sets of histone variants is developmentally regulated in a stage specific-manner. In unfertilized eggs and during the three initial cleavage divisions, chromatin is formed by cleavage stage (CS) variants. Then, from the 16 blastomer stage until hatching, alpha variants (α) are principally synthesized. From the blastula stage onward the late variants become predominant [Newrock et al., 1978; Maxson et al., 1983; Busslinger and Barberis, 1985; Imschenetzky et al., 1986; Von Holt et al., 1989]. Finally at the larval stages, somatic histones are the major components of chromatin, while the histone variants particular to earlier stages become minor components [Imschenetzky et al., 1993]. These changes in histone composition are reflected in differences in nucleosome populations [Shaw et al., 1981; Richards and Shaw, 1984]. The smallest nucleoprotein particles are found in unfertilized eggs and at the initial cleavage stages [Shaw et al., 1981]. These particles are formed by CS histone variants that interact with 129 bp of DNA in contrast to the 146 bp of DNA that are wrapped around a histone octamer in typical nucleosomes [Kornberg, 1977; Imschenetzky et al., 1989].

At present, the CS histone variants are not well characterized as compared to the early and late histone variants [Brandt et al., 1979; Kedes, 1979; Busslinger et al., 1985; Poccia, 1986]. These variants were first described by Newrock et al. [1978], and designated according to their electrophoretic migration related to histones from other sources. Then, seven major fractions were purified from unfertilized eggs of the sea urchin *Tetrapygus niger* by preparative electrophoresis. These fractions co-migrated with histones from other sources, but differed in amino acid composition. Due to the lack of correlation between each CS variant and the homologous histone of each class, these proteins were designated chromosomal proteins CSA-CSG [Imschenetzky et al., 1986]. The differences found between CS variants and somatic histones at larval stages of development were further supported by the immunological unrelatedness of these two sets of histone variants [Imschenetzky et al., 1993].

Two-dimensional electrophoretic analysis has shown that CS variants are heterogeneous [Poc-

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cia, 1986]. Based on immunobiochemical detection of poly(ADP-ribosylated) CS histone variants we have previously suggested that this heterogeneity may be due to the extensive poly-(ADP-ribosylation) of these proteins. We have also shown that this post-translational modification is a pre-requisite for S phase in zygotes [Imschenetzky et al., 1991a; 1992; 1993].

It is well established that this post-translational modification induces a great degree of electrophoretic heterogeneity. This is reflected by a family of subbands which migrate depending on the length of ADP-ribose moiety bound to the protein [Huletsky et al., 1985; Althaus and Richter, 1987; Boulikas, 1990; Boulikas et al., 1990]. Consequently, in order to precisely define the CS histone variants, this modification must be eliminated. Here we describe a procedure for hydrolysis of the ADP-ribose moiety from CS histone variants. In addition, we report that the elimination of the polymers of ADP-ribose reduces the heterogeneous native CS histone variants to five major proteins.

METHODS

Unfertilized Eggs and Zygotes of Sea Urchins

Sea urchin *Tetrapygus niger* was collected at the bay of Concepción, Chile, and maintained at room temperature under constant aeration in an aquarium containing natural sea water. Eggs and zygotes were obtained as described previously [Imschenetzky et al., 1980].

Isolation of CS Histone Variants From Chromatin and Gel Electrophoresis

CS histone variants were isolated from chromatin obtained from unfertilized eggs or from zygotes as described previously [Imschenetzky et al., 1980]. Untreated CS histone variants, as well as those recovered after performing different treatments to remove the ADP-ribose moiety, were analyzed by one-dimensional 18% (w/v) polyacrylamide gels containing sodium dodecyl sulfate (PAGE/SDS) as described by Laemmli [1970]. The electrophoretic analysis of these proteins in two-dimensional gels (2D PAGE) was performed in 12% w/v polyacrylamide gels containing 0.38% v/v Triton DF-16 and 8 M urea in the first dimension (PAGE/TAU) [Alfageme et al., 1974] and 18% w/v PAGE/SDS in the second dimension.

Detection of the ADP-Ribose Moiety of Native CS Histone Variants: Western Blots and Staining With Dansyl Hydrazine

CS variants were separated on PAGE/SDS, transferred to nitrocellulose membranes, and detected with polyclonal antibodies against polymers of ADP-ribose. The electrophoretic transfer and immunoblot analysis were performed by the procedure described by Towbin et al. [1979]. The serum anti-ADP-ribose polymers was obtained as described previously [Imschenetzky et al., 1991a,b].

To stain the ribose residues of the ADP-ribose moiety bound in vivo to CS variants, PAGE/SDS gels were treated with 0.5% w/v periodic acid which oxidizes the 2′ 3′ hydroxyl groups of the ribose molecule present in the linear polymers of the ADP-ribose. The aldehydes resulting from this oxidation were detected with dansyl hydrazine [Gander, 1984]. A violet contaminant present in commercially available dansyl hydrazine was removed by chromatography in silica gel 60 (Merck, Darmstadt, Germany). This violet compound stained all proteins in a nonspecific manner.

Labeling of ADP-Ribosylated CS Histone Variants In Vivo

To label the poly(ADP-ribose) moiety attached to CS histone variants in vivo, zygotes were incubated continuously from 3 to 90 min postinsemination (p.i.) in sea water containing 350 μCi/ml of 3H-adenosine (NEN, Boston, MA, Sp. act. 59 Ci/mmol). At 90 min p.i. these zygotes were collected and the labeled poly(ADP-ribose) moiety was detected by fluorography as described previously [Imschenetzky et al., 1992].

Hydrolysis of the ADP-Ribose Moiety of CS Histone Variants: Chemical Treatment With Hydroxyl Amine and Alkali

Enzymatic digestion with phosphodiesterase. To hydrolyze the ADP-ribose moiety bound to CS histone variants, native proteins were treated with hydroxyl amine or alkali as described by Burzio et al. [1979] with minor modifications. The samples containing poly-(ADP-ribosylated) CS variants were dissolved in 50 mM of a buffer Tris/HCl pH 7.5 and incubated in 1.0 M NH2OH at 37°C for 60 min or, alternatively, in 0.4 M NaOH at 56°C for 60 min.

The enzymatic treatment with phosphodiesterase was performed as described by Wesierska-Gadek and Savermann [1988]. In a typical ex-

periment, 300 μg/ml of native CS variants were incubated at 37°C for different times in a solution containing 300 μg/ml of snake venom phosphodiesterase I (Worthington, Freehold, NJ) dissolved in 50 mM Tris/HCl, pH 8.0, 4 mM urea, 2 mM NaCN, and a cocktail of protease inhibitors containing: 2 mM phenylmethylsulfonilfluoride (PMSF), 2 μg/ml antipain, 100 μg/ml N-tosyl-L-phenylalanine chloromethylketone (TPCK), and 50 μg/ml Nα-p-tosyl-L-lysine chloromethylketone (TLCK). To avoid solubility problems of the sample the digestion with PDE was performed at pH 8.0 instead of pH 8.9 as recommended for the commercial enzyme. Under these conditions no significant loss of enzyme activity was observed after 24 h of incubation. Following each incubation, the acid soluble proteins were extracted with 0.25 N HCl for 2 h and the supernatant containing the treated samples was precipitated with 20% w/v TCA, washed once with acetone 0.07 N HCl and several times with cold acetone. The treated samples were vacuum dried and stored at −20°C.

Coupling of Phosphodiesterase (PDE) to CNBr-Sepharose 4B

One milligram of snake venom phosphodiesterase I (Worthington) was dialyzed against a solution containing 0.1 M NaHCO3 pH 8.3 and 0.5 M NaCl. The PDE solution was incubated overnight at 4°C with 1 g of CNBr-activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden). To block the remaining reactive groups the Sepharose-phosphodiesterase (S-PDE) was transferred to 0.2 M glycine, pH 8.0. This treatment was repeated three times. To remove the uncoupled protein, the S-PDE beads were washed in 0.1 M NaHCO3, pH 8.3, then in 0.5 M NaCl and finally in a solution containing 1 M KCl, 0.2 M glycine adjusted to pH 8.0. This washing procedure was repeated until no detectable amount of protein was released from the S-PDE. Generally, this procedure was performed three or four times. The resulting S-PDE was stored in suspension at 4°C. This procedure couples about 45 ± 3% of the protein contained in the PDE extract to the sepharose. The S-PDE obtained retained about 30% of the total activity of the soluble PDE. No significant loss of activity of the immobilized enzyme was observed after 24 h of incubation at 37°C. The S-PDE obtained was stable for several months stored at 4°C.

Digestion of the (ADP-Ribose) Moiety of CS Histone Variants With S-PDE

Three hundred micrograms of native CS histone variants, unlabeled or labeled with ^{3}H-adenosine, were dissolved in a solution of 20 mM Tris/HCl, pH 8.0, containing 4 mM urea, 2 mM NaCN, and a cocktail of proteases inhibitors (2 mM PMSF, 2 μg/ml antipain, 100 μg/ml TPCK, and 50 μg/ml TLCK). Five hundred microliters of the S-PDE (1.8 U of PDE activity) was then added to this solution. After incubation at 37°C for different periods of time, the S-PDE was recovered by centrifugation. To remove the adsorbed CS variants, the gel was washed with a solution containing 1 M KCl and 0.2 M glycine adjusted to pH 8.0. The supernatant, containing the CS histone variants, was made 0.25 N in HCl and precipitated with 20% w/v TCA. The resulting pellet was washed once with acid acetone (0.07 N HCl) and twice with cold acetone. The proteins obtained were dried under vacuum and stored at −20°C.

RESULTS

Detection of the ADP-Ribose Moiety of CS Histone Variants

Whole CS histones isolated either from the chromatin of unfertilized eggs (Fig. 1, lanes 1 and 5) or zygotes (Fig. 1, lane 2) were separated by one-dimensional polyacrylamide gel electrophoresis (SDS/PAGE), transferred to nitrocellulose membranes, and analyzed by Western blot with polyclonal antibodies directed against the ADP-ribose moiety. As shown in Figure 1A, CS histone variants have bound polymers of ADP-ribose in vivo, both in unfertilized eggs and in zygotes. The CS variants isolated from unfertilized eggs (Fig. 1, lane 3) and those purified from zygotes (Fig. 1, lane 4), exhibited strong positive signals located at 56 and 29 KD and diminished signals located at 50, 17, and 14 KD. The poly(ADP-ribosylation) of CS histone variants was further confirmed by the incorporation of ^{3}H-adenosine into CS variants during the initial cleavage stages of sea urchin development. The resulting ^{3}H-adenosine labeled CS histone variants were revealed by fluorography (Fig. 1B, lane 6).

To establish a more direct relationship between the electrophoretic heterogeneity of native CS variants and the presence of chains of ADP-ribose on these proteins, whole CS variants isolated from zygotes harvested 90 min p.i.

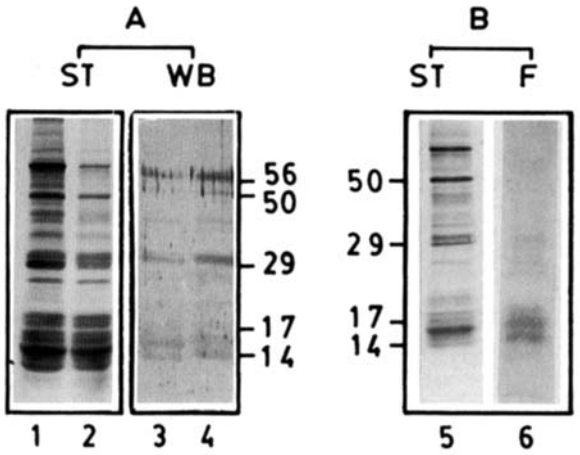

Fig. 1. Detection of the ADP-ribose moiety in native CS histone variants: **A:** Western blot analysis of the ADP-ribose moiety of CS histone variants from unfertilized eggs and zygotes. Sixty micrograms of CS variants were separated in SDS/PAGE, transferred to nitrocellulose, and revealed using rabbit anti-poly (ADP-ribose) sera as described in Methods. Coomasie blue stained gels (ST) are compared with the corresponding Western blots (WB). CS histone variants isolated from unfertilized eggs (*lanes 1* and *3*) and from zygotes (*lanes 2* and *4*). **B:** Incorporation of ^{3}H-adenosine into the ADP-ribose moiety of CS histone variants in early embryos. Whole CS histone variants were isolated from two blastomers embryos harvested 120 min postinsemination (p.i.). Sixty micrograms of these proteins were separated in SDS/PAGE and the ^{3}H-adenosine labeled poly(ADP-ribosylated) CS histone variants were analyzed by fluorography as described in Methods. Coomasie blue stained sample (*lane 5*), fluorography (*lane 6*).

were separated in 2-D gels and treated with periodic acid and dansyl hydrazine as described in Methods. This treatment detects the ribose residues of the ADP-ribose moiety; therefore, it is possible to detect oligomers that would otherwise remain undetectable. As previously shown, anti(ADP-ribose) sera reacts mainly with chains over 18–20 mer; consequently short oligomers are undetectable by Western blot analysis [Kanai et al., 1977, 1978; Imschenetzky et al., 1991a]. The treatment with dansyl hydrazine shows the presence of ADP-ribose chains on the majority of Coomasie stained native CS variants, confirming that the electrophoretic heterogeneity of CS histones is due to poly(ADP-ribosyl)ation of these proteins (Fig. 2A and B).

It has been reported that ADP-ribose polymers can be noncovalently bound to chromosomal proteins in vivo by protein glycation [Kiehlbanch et al., 1993]. This glycated moiety of proteins is unstable if the modified proteins are incubated at pH 9.0 [Cervantes-Laurean et al., 1994]. To avoid this possibility, poly(ADP-ribosyl)ated CS variants, labeled in vivo with ^{3}H-adenosine, were incubated in a buffer containing 0.5 M Tris, pH 9.0, 50 mM $MgCl_2$. After 2 h of incubation the remaining radioactivity bound to the CS histone variants was measured. The results shown in Table I demonstrate that the labeled polymers are stable at pH 9.0. This result strongly suggests that protein glycation is not involved in the binding of ADP-ribose to the CS variants in vivo. In addition, we observed that in this procedure markedly decreased the solubility of labeled samples (not shown).

From these results we have concluded that the electrophoretic heterogeneity of CS histone variants is mainly due to the extent that these proteins are poly(ADP-ribosyl)ated in both unfertilized eggs and during the two initial cleavage divisions of sea urchins.

Chemical Treatments to Remove the ADP-Ribose Moiety From Native CS Histone Variants

To eliminate the ADP-ribose moiety from the CS histone variants, these proteins were treated with 1 M hydroxylamine or alternatively with 0.4 M sodium hydroxide. The treated samples were then analyzed by electrophoresis in 2D PAGE. When treated and untreated proteins were compared, it was found that the treatment with NH2OH did not alter the 2D patterns of electrophoretic migration of CS variants from unfertilized eggs (Fig. 3, compare A and B) or from zygotes (Fig. 3, compare C and D). On the other hand, following alkali treatment most of the CS proteins were found as an insoluble mixture that did not enter the gel, or alternatively, the proteins that enter the gel migrated as a smear of unresolved heterogeneous low molecular weight molecules (Fig. 3, compare E and F). On the basis of these results it was concluded that most of the ADP-ribose moieties bound to the CS histone variants are resistant to NH2OH and that the incubation with alkali appears to be deleterious to these proteins.

Enzymatic Degradation of the ADP-ribose Moiety of CS Histone Variants: Treatment With Soluble and Immobilized Phosphodiesterase

To remove the ADP-ribose moiety from CS histone variants, these proteins were isolated from unfertilized eggs and incubated with snake venom phosphodiesterase for 2 h. Then, the reaction mixture was made 0.25 N in HCl and the acid insoluble material was removed a low speed centrifugation. The remaining basic proteins were then recovered and analyzed by SDS/

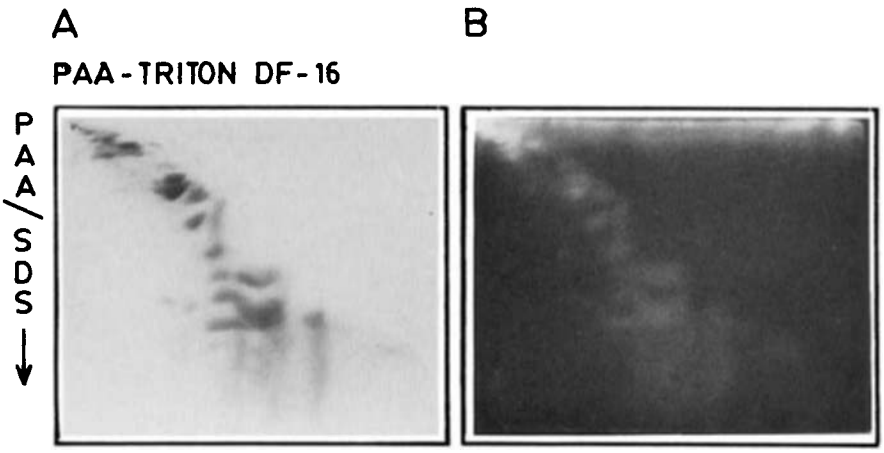

Fig. 2. Detection of the ADP-ribose moiety in native CS histone variants by its reaction with dansyl hydrazine. A sample of 150 μg of CS histone variants isolated from 2 blastomers embryos harvested 120 min p.i. was analyzed in 2-D PAGE. This gel was first treated with dansyl hydrazine as described in Methods. The fluorescent compound resulting after this treatment was visualized with UV light and photographed (**B**). The dansyl hydrazine treated gel was subsequently stained with Coomasie blue (**A**).

TABLE I. Treatment at pH 9.0 of ^{3}H(ADP-Ribosylated) CS Histone Variants*

Hours of treatment	Remaining radioactivity (cpm)
Untreated	5,220
2 h	5,020

*The ADP-ribose moiety of CS histone variants was labeled in vivo with ^{3}H-adenosine. The labeled CS histone variants were isolated from zygotes harvested 120 min postinsemination and incubated at pH 9.0. The label remaining after this incubation was measured as described in Methods.

PAGE. An aliquot containing the acid soluble proteins present in the extract of phosphodiesterase I was analyzed in parallel. As shown in Figure 4, the PDE digestion of CS histone variants resulted in a marked reduction of the electrophoretic heterogeneity of these proteins (Fig. 4, lanes A and C). We also observed that the acid soluble fraction of the PDE extract contains several peptides which migrate similarly to the undigested CS variants (Fig. 4B). Consequently we concluded that although the ADP-ribose moiety of CS variants is efficiently removed by phosphodiesterase I, the acid soluble peptides present in the enzyme extracts complicates the interpretation of the results. To overcome this problem, PDE coupled to activated Sepharose 4B (S-PDE) was used instead of the soluble PDE extract. The digestion efficiency of this system was determined by incubating ^{3}H-adenosine labeled CS variants with S-PDE. As shown in Table II, after 2 h of incubation the vast majority of the label was released from the treated CS proteins. The residual radioactivity was not reduced significantly by longer digestion, suggesting that this remaining label may correspond to mono ADP-ribose, which is known to be resistant to this enzyme.

Furthermore the removal of the ADP-ribose moiety from the CS histone variants was also confirmed by the substantial decrease in heterogeneity of CS histone variants after incubation with S-PDE. A comparison of the electrophoretic migrations in two-dimensional PAGE-SDS of untreated and S-PDE treated sample is shown in Figure 5. The heterogeneous and complex electrophoretic pattern of the untreated CS fractions resolved into five major spots in the treated samples (Fig. 5A and B).

DISCUSSION

The evidence presented in this report confirms our previous suggestions that CS histone variants are extensively poly(ADP-ribosylated) in zygotes of sea urchins as well as CS variants present in unfertilized eggs (Imschenetzky et al., 1991a; 1992). Our conclusion is based on three major lines of evidence: (1) the incorporation of ^{3}H-adenosine in vivo into poly(ADP-ribosylated) CS histone variants during the initial cell cycles of sea urchin zygotes; (2) the immunodetection of the poly(ADP-ribose) chains with polyclonal antibodies elicited against polymers of ADP-ribose; and (3) the detection of the ribose residues present in the poly(ADP-ribose) moiety by its reaction with dansyl hydrazine.

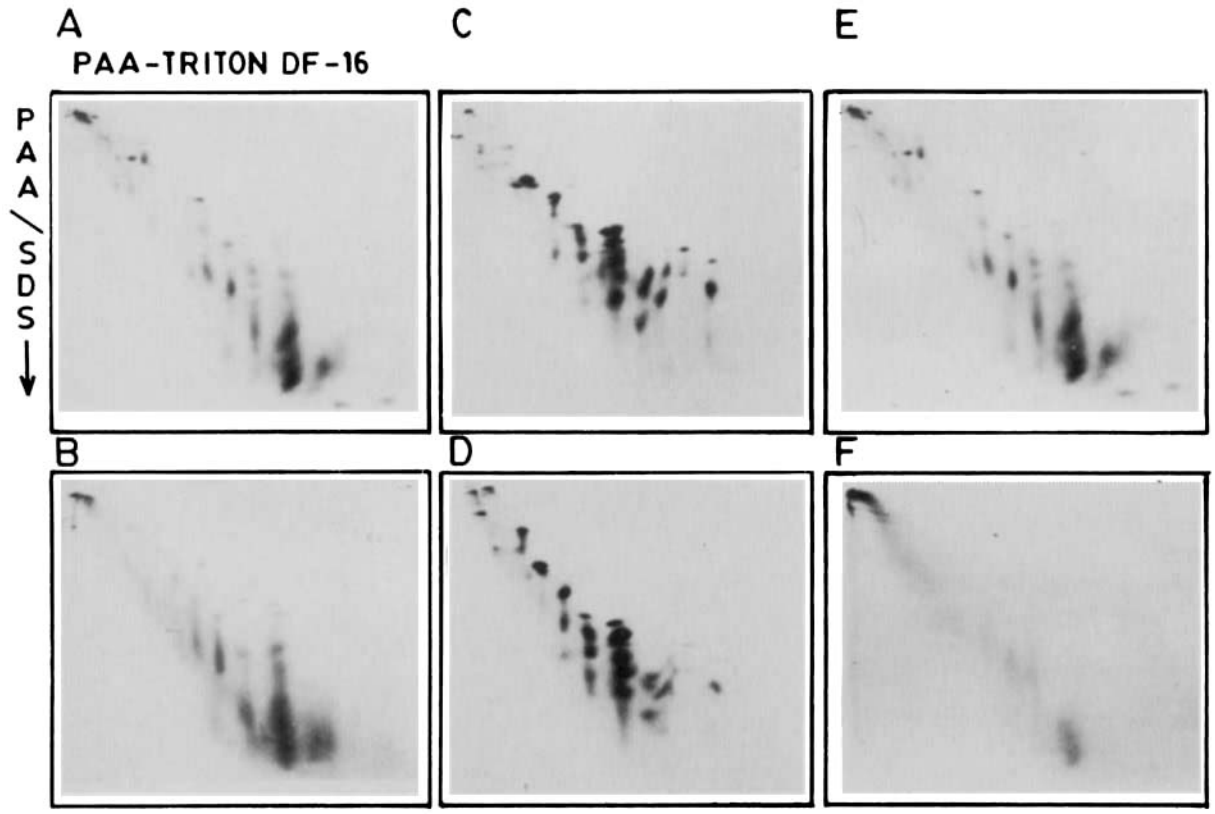

Fig. 3. Chemical treatments performed to hydrolyze the ADP-ribose moiety of native CS histones. Five hundred micrograms of whole CS histone variants were isolated from unfertilized eggs or from zygotes and subjected to treatments with hydroxylamine and sodium hydroxide as described in Methods. Subsequently, 150 μg of treated samples were analyzed by 2D-PAGE and the Coomasie blue stained patterns obtained were compared with that of 150 μg of untreated samples analyzed in 2-D PAGE. Coomasie stained CS histone variants from unfertilized eggs: untreated (**A** and **E**), treated with 1 M NH2OH (**B**) or with 0.4 M NaOH (**F**). Coomasie stained CS histone variants from zygotes: untreated (**C**) and treated with 1 M NH2OH (**D**).

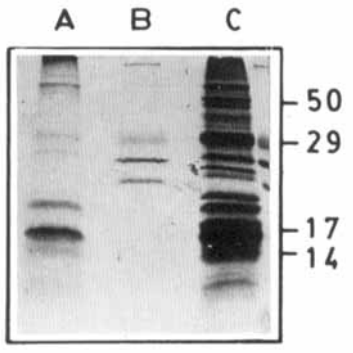

Fig. 4. Enzymatic hydrolysis of the ADP-ribose moiety of CS histone variants with soluble phosphodiesterase (PDE). Three hundred micrograms of unfertilized eggs histone variants were incubated for 2 h with PDE, the resulting acid soluble proteins were analyzed in SDS/PAGE. Coomasie blue stained bands of untreated CS histone variants (*lane C*) is compared with the PDE treated sample (*lane A*) and with the acid soluble peptides present in the PDE extract (*lane B*).

TABLE II. Hydrolysis of ADP-Ribose Moiety of CS Histone Variants With Sepharose-Phosphodiesterase (S-PDE)*

Treatment (h)	Remaining radioactivity (cpm)
0	12,500
2	1,550
4	1,470

*The ADP-ribose moiety of CS histone variants was labeled in vivo with ^{3}H-adenosine. Whole ^{3}H-adenosine poly(ADP-ribosylated) CS histone variants were isolated from zygotes harvested 120 min postinsemination and hydrolyzed with S-PDE. The radioactivity remaining after this treatment was measured as described in Methods.

In contrast to the most common poly(ADP-ribosyl)ation found in histones from other sources, CS histone variants are not substantially modified in glutamic residues in vivo. This conclusion is based on the finding that the majority of the poly(ADP-ribosylated) forms of native CS histones were not altered after incubation

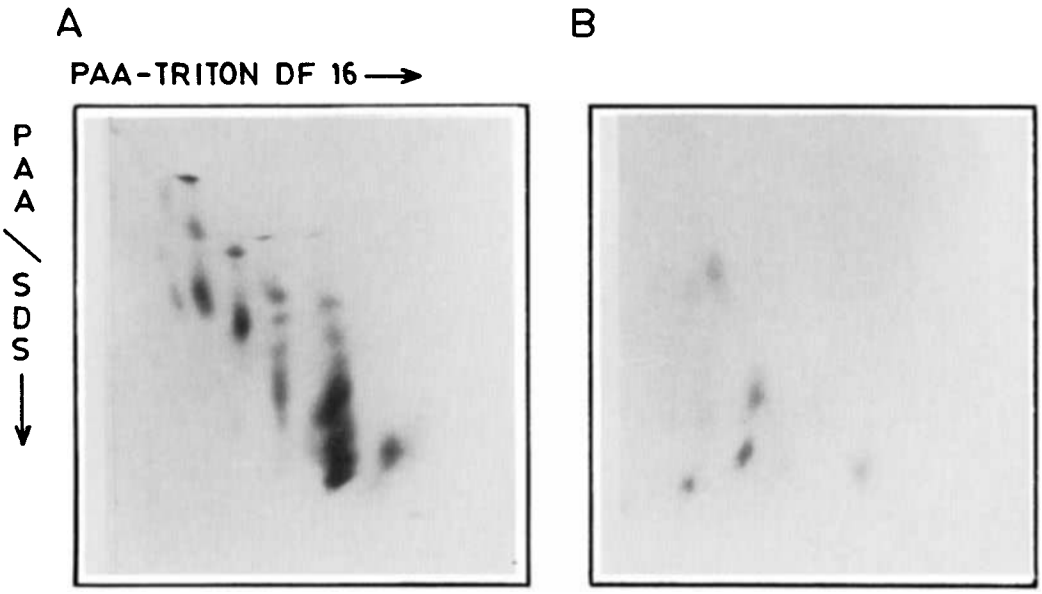

Fig. 5. Electrophoretic migration of CS histone variants after the hydrolysis of the ADP-ribose moiety with phosphodiesterase bound to Sepharose (S-PDE). Five hundred micrograms of unfertilized eggs histone variants were incubated for 2 h with S-PDE, the S-PDE was removed by low speed centrifugation, and 100 μg of the resulting acid soluble proteins were analyzed by 2-D PAGE as described in Methods. Untreated samples (**A**) and S-PDE treated samples (**B**).

with hydroxylamine, which is the standard procedure to hydrolyze the ADP-ribose chains bound to the glutamic residues of proteins [Bredehorst et al., 1978; Gaal and Pearson, 1985]. Alternatively a noncovalent binding of ADP-ribose polymers to CS histone variants through a protein glycation is unlikely, since the ADP-ribose moiety of CS histone variants was stable at pH 9.0. As communicated recently, ADP-ribose polymers bound to proteins by glycation are readily released from proteins under this condition [Cervantes-Laurean et al., 1994].

In this report we have demonstrated that the electrophoretic heterogeneity previously described for native CS variants in two-dimensional gels is mainly due to different extents of poly(ADP-ribosylation) of five major proteins. These five proteins were detected by 2D-PAGE after S-PDE digestion of whole native CS histone variants. A clear correlation of each one of these proteins with histones from other sources can not be established thus far. As reported, CS variants are immunologically unrelated with somatic type histones found in larval stages of development [Imschenetzky et al., 1993]. In addition, antiserum against sperm-specific histones do not cross-react with CS histone variants, except for a single CS fraction that exhibits a weak cross reactivity with sperm H2A, but migrates differently when analyzed in 2D-PAGE [Zweidler, 1978; Imschenetzky et al., 1991b].

Despite the immunological difference between the native CS histone variants and histones from other sources, we postulate that CS variants should be considered as genuine histones in functional terms. The main considerations to homologue the function of CS histone variants with histones can be summarized as follows: CS variants pack the DNA into small nucleo-protein particles which in turn are further organized into higher ordered structures [Imschenetzky et al., 1989a]. CS variants are extensively poly(ADP-ribosylated) in response to embryonic demands for DNA replication and/or DNA repair [Imschenetzky et al., 1991a; 1992, 1995a]. The synthesis de novo of the CS variants is confined to the S phase of the cell cycle in early zygotes and the newly synthesized CS variants are immediately targeted to nuclei and bound to DNA [Imschenetzky et al., 1995b], as well as histones from most cells [Stein et al., 1994].

The immunological nonrelatedness of CS variants and somatic histones implies a lack of homology between the genes that encode these proteins and those encoding early or late histone variants. CS histone variants are the only set of variants that remain unknown among the well-defined family of developmentally regulated histone genes from sea urchins [Kedes, 1979; Maxson et al., 1983; Busslinger and Barberis, 1985; Kaumeyer and Weinberg, 1986; Childs et al.,

1982; Kemler and Busslinger, 1986; Knowles et al., 1987]. A new challenge is presented by the molecular characterization and isolation of the genes encoding the non ADP-ribosylated forms of CS histone variants, a less complex and more defined set of proteins.

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