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# LOCALIZATION OF PROTEOGLYCANS IN THE CELL NUCLEUS OF SEA URCHINS AS REVEALED BY MEANS OF LECTIN-BINDING AND NUCLEASE DIGESTION

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## SUMMARY

The distribution of proteoglycans in the embryonic cell nucleus of sea urchins was studied by means of lectin binding using *Ricinus communis* agglutinin I (RCA-120) and other lectins. By electron microscopy using avidin-biotin complex technique, the localization of nuclear proteoglycans was found to be restricted exclusively to areas of dispersed chromation, i.e. euchromatic regions, in the uncleus. Fluorometric determination of lectin binding ability onto the partially digested chromatin using FITC-labeled lectins showed that lectin binding sites were exposed by the action of pancreatic DNase-I but remained masked by Staphylococcal nuclease. Considering the difference of the mode of action of these enzymes, it is likely that the lectin binding sites are more abundant in the regions of active genes than those of inactive ones. The pattern of localization of the nuclear proteoglycans suggests a possible participation of these substances in the mechanism of gene activation.

Nuclear proteoglycans have been suggested to play a crucial role in determining the fate of differentiation in embryonic cells [1-10], presumably by modifying the minute structure of the chromatin [4,5]. However, we are still quite ignorant about the localization of proteoglycans within the nucleus.

In the present study, the localization of nuclear proteoglycans was examined from two different viewpoints. First the manner of distribution of proteoglycans was compared electron microscopically between dispersed and condensed areas of the chromatin in the nucleus, in which experiment the location of proteoglycan was visualized by means of the technique of indirect ferritin-labeling, using biotinyl lectin and avidin-conjugated ferritin [11,12]. Second, the lectin-binding ability was compared between partially digested chromatin preparations using pancreatic DNase-I on the one hand and bacterial nuclease on the other, taking advantage of preferential disintegration of euchromatic regions by the former enzyme [13,14] and indiscriminate digestion by the latter [15-18].

It was demonstrated in the present experiment that distribution of the nuclear proteoglycans was restricted to the area of dispersed chromatin in the nucleus, and that considerable amounts of lectin-binding sites were unmasked by the action of DNase-I, whereas no unmasking was observed in the case of bacterial nuclease. These facts seem to indicate the partial distribution of lectin-

binding sites in the chromatin. It would be of interest if the distribution of the lectin-binding sites, detected either morphologically or biochemically, showed some correlation with the location of genetically active regions in the nucleus. This would suggest a possibility that nuclear proteoglycans may play a regulating role in the mechanism of gene activation.

## MATERIALS AND METHODS

*Materials*:

The sea urchins, *Clypeaster japonicus* and *Hemicentrotus pulcherrimus*, were used as materials. Eggs and sperm were secured by introduction of isotonic KCl into the body cavity of the animals. Eggs were washed with filtered sea water, inseminated, and allowed to develop at 20°C with stirring until the stage of mesenchyme-blastula.

*Isolation of nuclei*:

Embryos were packed by gentle centrifugation. They were dissociated and the extracellular substances were removed in 1M glycine-2mM EDTA (pH 8.3) at 0°C by stroking in a Dounce type homogenizer driven by hand. Then, blastomeres were disrupted for isolation of nuclei by homogenization in 10% Percoll (Pharmacia, Uppsala)-10mM Tris-HCl buffer (pH 7.4) containing 1mM phenylmethyl sulfonyl fluoride (PMSF) with an usual Teflon-glass homogenizer. Percoll, composed of colloidal silica coated with polyvinylpyrrolidone, was used as a protective reagent for nuclear structures and at the same time as a reagent for preparing the density gradient in the later steps of nuclear purification. The use of sugars such as sucrose was avoided in the preparation of nuclei, since these substances may interfere with the lectin binding reaction [10]. PMSF was added in order to prevent degradation of nuclear proteoglycans.

Crude nuclei were sedimented by centrifugation at 2,000 rpm for 10min, resuspended in 10% Percoll-10mM Tris-HCl buffer (pH 7.4) The contaminated cytoplasmic debris was removed by centrifugation at 3,000 rpm for 20 min through a column of linear density gradient made by Percoll (10%-25%). The nuclei were further purified by shearing in 0.1% Triton X-100 in 10mM Tris-HCl buffer (pH 7.4) using a Dounce type homogenizer. The nuclei were freed from their outer membrane together with adherent traces of cytoplasmic debris by this procedure. Triton X-100 was washed out thoroughly with 10mM Tris-HCl buffer (pH 7.4) immediately after the treatment. The washing was repeated three times, and the nuclei were sedimented once at 7,000 rpm and twice at 11,500 rpm. The purity of nuclei was checked by estimating protein/DNA ratio, and preparations which showed a value higher than 2.0 were discarded as samples of inadequate purity. Usually values between 1.5 and 1.9 were obtained.

*Treatment of nuclei with biotinyl lectin and ferritin-labeled avidin for electron microscopy*:

For the detection of proteoglycans in electron microscopy, *Ricinus communis* agglutinin I (RCA-120) was chosen as the probe lectin, [9]. Isolated nuclei were prefixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 20min at room temperature, and rinsed twice with the saline buffer solution composed of 0.15M NaCl-50mM Tris-HCl (pH 7.4). Nuclei were incubated with biotinyl RCA-120 (Vector Laboratories, Burlingame, CA), at a concentration of 500$\mu$g/ml in the saline buffer at 0°C overnight, in order to modify the nuclear proteoglycans by binding with the

lectin. The nuclei were rinsed with two changes of the saline buffer to wash out the unbound biotinyl RCA-120 and then incubated in 500$\mu$g/ml ferritin-avidin D conjugate (Vector Laboratories) in the saline buffer at 4°C overnight by which procedure the proteoglycans were labeled intensely with ferritin grains. After incubation, the nuclei were rinsed with two changes of the saline buffer.

As a control experiment, the nuclei were incubated at 0°C for 30min with $\alpha$-D-galactose as a competitive inhibitor of RCA binding, prior to the reaction with biotinyl RCA-120 to verify that the binding of RCA was of a lectin-specific nature. The nuclei were also incubated solely with ferritin-avidin conjugate, omitting the incubation with biotinyl RCA-120 to exclude the possibility of direct binding of this reagent with the chromatin.

For electron microscopy, the nuclei were re-fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) and then post-fixed in 1% osmium tetraoxide in the same buffer. After dehydration through a graded series of ethanol and acetone, the specimens were embedded in Epon 812 resin for sectioning. The sections were double stained with uranyl nitrate and lead citrate, unless otherwise stated. Observations were done using a JEM-1200EX electron microscope.

*Limited digestion of chromatin for estimation of lectin-binding ability by fluorometry*:

The nuclei were digested with pancreatic DNase- I (grade II, Boehringer, Mannheim) which disintegrates the euchromatic region preferentially [13,14]. For comparison, they were also digested with Staphylococcal nuclease (Boehringer) under the same conditions, which enzyme disintegrates inter-nucleosomal connections overall the chromatin indiscriminately [15-18]. Only partial digestion by these enzymes was allowed by limitting the enzyme concentration and incubation time. Partially digested chromatin was allowed to bind with the fluorescein-labeled lectins (FITC-lectins), and the amount of bound lectin was measured fluorometrically.

The reaction mixture (1.2ml in total) contained 60$\mu$g nuclei as DNA, 120 units of DNase-I or nuclease, 100$\mu$g FITC-lectins in 150mM NaCl-50mM Tris-HCl (pH 7.4), unless otherwise stated. Digestion of chromatin was started in a chilled chamber by adding the enzyme and terminated by sedimentation of nuclei at 20,000 rpm for 10min, according to the protocol as shown in Table 1. The reaction mixture was agitated mechanically during the course of incubation by sonication using an ultrasonic cell disrupter (Tomy Seiko, Tokyo) at a setting of 80W for 2sec five times (10sec in total), while maintaining the same temperature.

**Table 1.** Protocol for the limited digestion of nuclei by nucleotidases and binding of FITC-lectins onto the exposed proteoglycan-sites.

Time*
−90min Preincubation of nuclei with FITC-lectin started.
−60min Preincubation continued with additional blocking sugars in one reaction tube of the duplicate.
0min Digestion of nuclei with nucleotidase started by adding the enzyme.
15min Reaction mixture sonicated.
30min** End of incubation. Partially digested nuclei sedimented by centrifugation at 20,000 rpm for 10min.
45min Nuclear sediment washed with lectin-free medium and sedimented by centrifugation.
60min Nuclear sediment digested with actinase at 37°C.

* Digestion of nuclei with nucleotidase was started at 0min.
** Longer incubation time was set according to the experimental schedule.

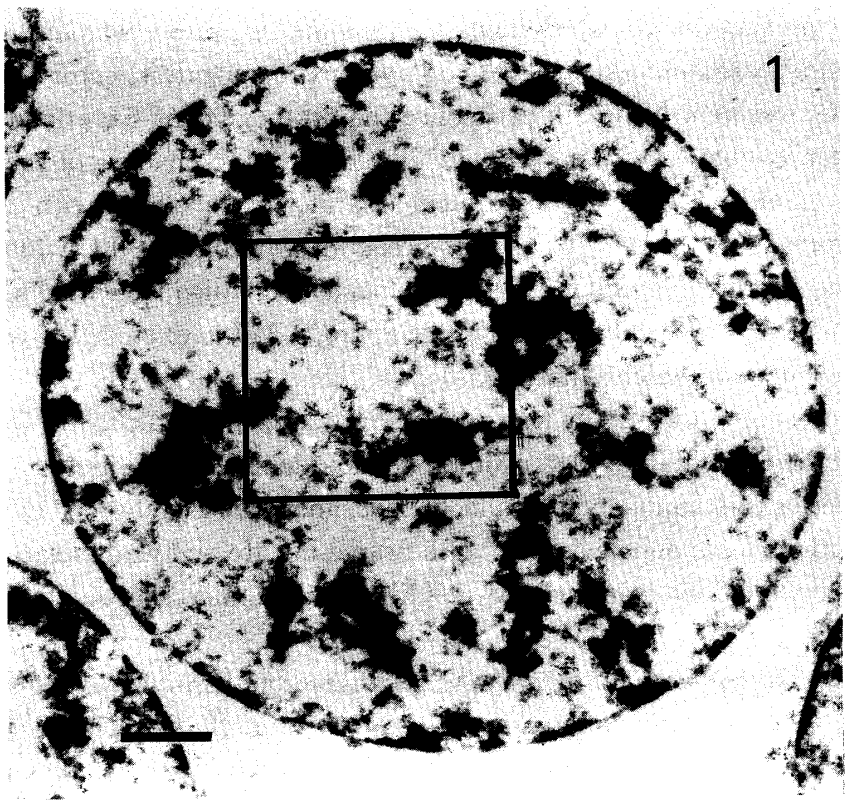


**Fig. 1.** Nucleus of sea urchin embryo treated with biotinyl RCA-120 followed by ferritin-avidin conjugate. Nuclei were isolated at the stage of mesenchyme blastule (*Hemicentrotus pulcherrimus*) . Bar: 500nm.

Sedimented chromatin was washed twice with the saline buffer without the lectins, and solubilized by digestion with actinase (50$\mu$g/ml) (Kaken Kagaku, Tokyo) at 30°C overnight. After removal of the insoluble part by centrifugation, the fluorescence intensity was measured using a fluorescence photometer at the excitation wave lenght of 493nm and emission wave lenght of 515nm. Duplicates were always run in parallel using the same batch of chromatin. The lectin binding was blocked by adding competitive sugars in one reaction tube, while the lectin was allowed to bind freely in the other tube. Subtraction of the fluorescence intensities between the two was taken as the value for the specific lectin binding.

The FITC-lectins used were *Ricinus communis* agglutin I (RCA-120), Concanavalin A (Con A), and wheat germ agglutinin (WGA), which were purchased from Seikagaku Kogyo, Tokyo. In the case of Con A, the salt composition of the reaction mixture was modified to contain 1mM $CaCl_2$ and 1mM $MnCl_2$ additionally. As the competitive sugars, D-galactose (for RCA-120), $\alpha$-methyl-D-mannoside (for Con A), and N-acetyl-D-glucosamine (for WGA) were used at final concentrations of 0.2M.

*Others*:

DNA was determined according to the method of Burton [19], and protein by the method of Lowry *et al.* [20].

## RESULTS

*Distribution of lectin-binding sites observed by electron microscopy*

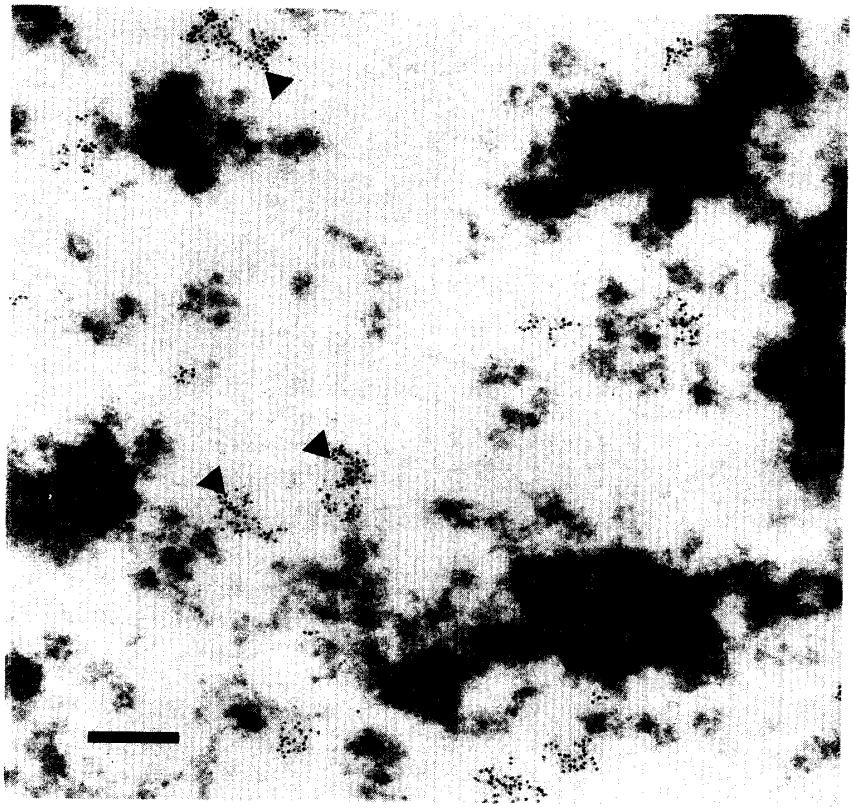

**Fig. 2.** The same preparation as Fig. 1 at a higher magnification. The square in Fig. 1 corresponds to the field in Fig. 2. Arrow-heads indicate clusters of ferritin grains. Note that the distribution of ferritin grains is restricted in the dispersed (euchromatic) area of chromatin network (*Hemicentrotus pulcherrimus*). Bar: 200nm.

Isolated nuclei were treated with biotinyl RCA-120 followed by ferritin-avidin D conjugate. By this indirect labeling procedure, the localization of nuclear proteoglycans was visualized by deposition of ferritin grains. As is seen in Figs. 1 and 2, ferritin grains were found to form clusters and their distribution was confined exclusively to the dispersed area of the chromatin.

The condensed area of the chromatin seemed to lack the lectin-binding sites. This was confirmed further in the preparations in which the step of the staining with uranyl nitrate and lead citrate was omitted (Fig.3). In these nuclei, the condensed chromatin was observed as a vague blur, and no ferritin grain was located on this structure. No ferritin grain was observed in the nucleolar regions (Fig. 4).

Figure 5 shows a nuclear preparation in which the binding reaction of RCA-120 was carried out in the presence of 0.4M galactose, one of the most potent competitive sugars for this lectin. No grain of ferritin was observed in any nuclear structure, including the dispersed chromatin region, suggesting that the binding of RCA-120 was of a specific nature due to the reaction between the lectin and polysaccharides. It was also confirmed that there occurred no direct dinding of ferritin-avidin D conjugate on the nuclear structures in the absence of previous treatment with biotinyl RCA-120.

*Change of lectin-binding ability in partially digested chromatin by pancreatic DNase-I and bacterial nuclease*

The structure of isolated nuclei were partially loosened by digestion with two kinds of nucleotidases accompanying mechanical agitation. Pancreatic DNase-I and Staphylococcal nuclease

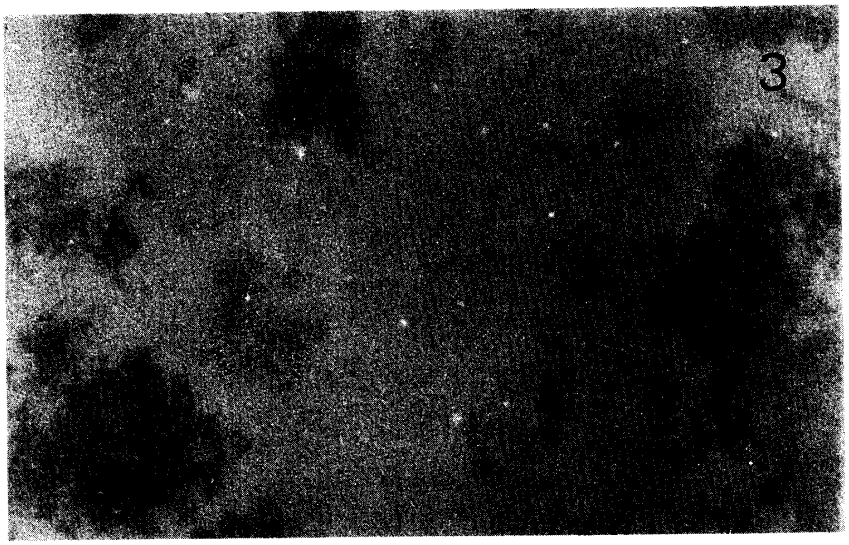

**Fig. 3.** The same batch preparation of nuclei as Fig. 2 except that the step of staining with uranyl nitrate and lead citrate was omitted. Condensed chromatin appeared as a vague blur. Note the absence of ferrition grain in condensed (heterochromatic) areas of the chromatin (*Hemicentrotus pulcherrimus*). Bar: 200nm.

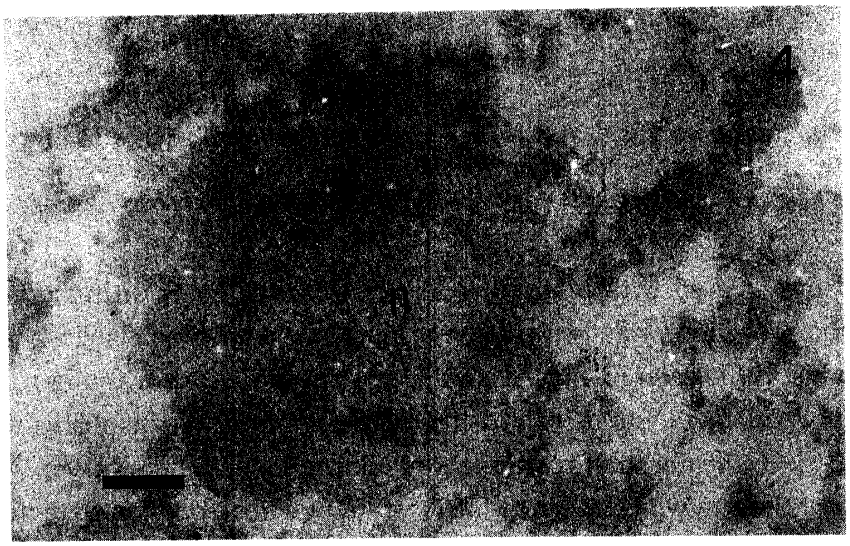

**Fig. 4.** Nucleolar region. The specimen was treated with biotinylated RCA-120 followed by ferritin-avidin conjugate but the step of staining with uranyl nitrate and lead citrate was omitted. No ferritin grain was observed in the nucleolus (*Hemicentrotus pulcherrimus*). n : nucleolus. Bar: 200nm.

were chosen on account of their different mode of action on chromatin. They were then allowed to bind with several kinds of fluorescein-labeled lectins, RCA-120, Con A, and WGA, making use of their affinity to different sugar residues of proteoglycans. The change of lectin-binding ability was determined fluorometrically by estimation of fluorescence intensity of the chromatin.

The results differed according to the kind of nucleotidase and also the kind of lectins used.

*RCA-120*: As is seen in Fig. 6, the native chromatin was revealed to show lectin-binding ability at a very low level. After the digestion by pancreatic DNase-I, the lectin-binding ability increased approximately threefold, while no increase was observed by the action of Staphylococcal nuclease. The result of digestion by the combination of the two enzymes was the same as that by DNase-I alone.

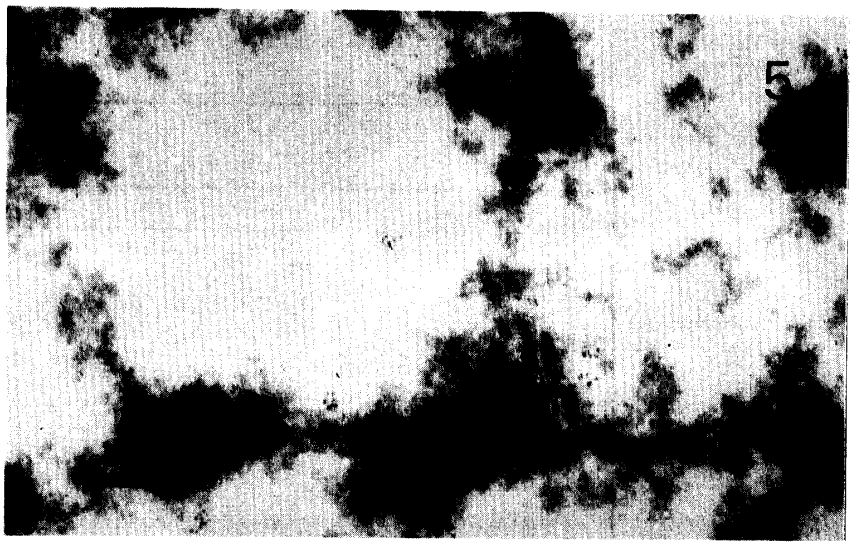

**Fig. 5.** Competitive inhibition of RCA-120 binding. Nuclei treated with biotinylated RCA-120 and ferritin-avidin in the presence of 0.4M galactose. Note that no ferritin grain was observed in either dispersed (euchromatic) or condensed (heterochromatic) areas in the nucleus (*Hemicentrotus pulcherrimus*). Bar: 200nm.

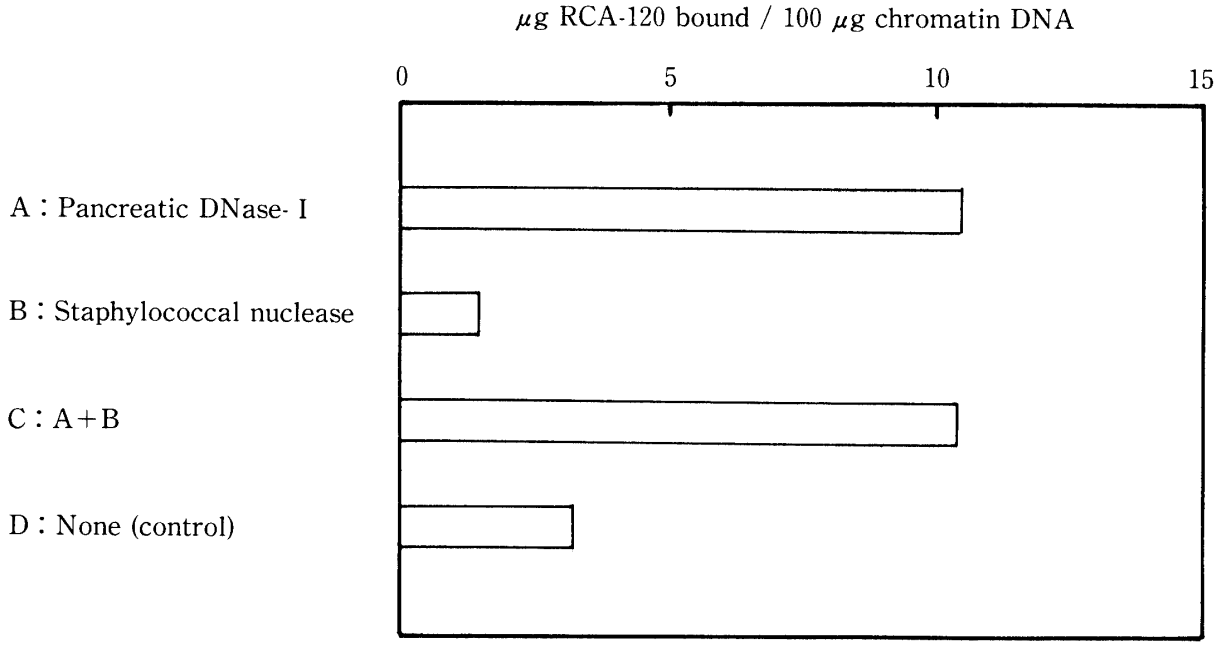


**Fig. 6.** Binding of RCA-120 with sea urchin chromatin after treatment with pancreatic DNase-I and Staphylococcal nuclease. Reaction mixture (1.2ml) contained 100μg FITC-RCA-120, 60μg chromatin (as DNA), and 120 units of the enzyme. (*Clypeaster japonicus*)

*Con A*: Similar results were obtained using Con A (Fig. 7). Considerable amounts of Con A-binding sites became apparent by the action of pancreatic DNase-I, while practically no increase in the lectin-binding ability was observed using Staphylococcal nuclease. The combination of the two enzymes gave the same result as that of DNase-I alone.

*WGA*: Native chromatin showed practically no binding sites for WGA, and no change in WGA-binding ability after digestion by the two nucleotidases, either given alone or in combination (Fig. 8).

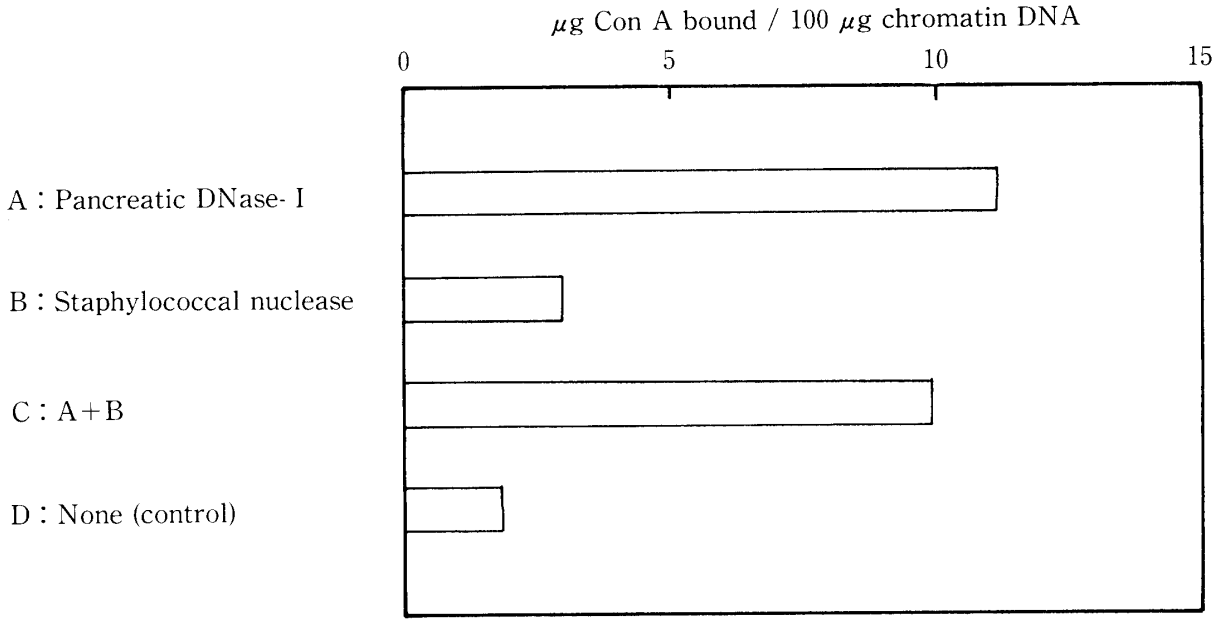


**Fig. 7.** Binding of Con A with sea urchin chromatin after treatment with pancreatic DNase-I and Staphylococcal nuclease. Reaction mixture (1.2ml) contained 100μg FITC-Con A, 60μg chromatin (as DNA), and 120 units of the enzyme. (*Clypeaster japonicus*)

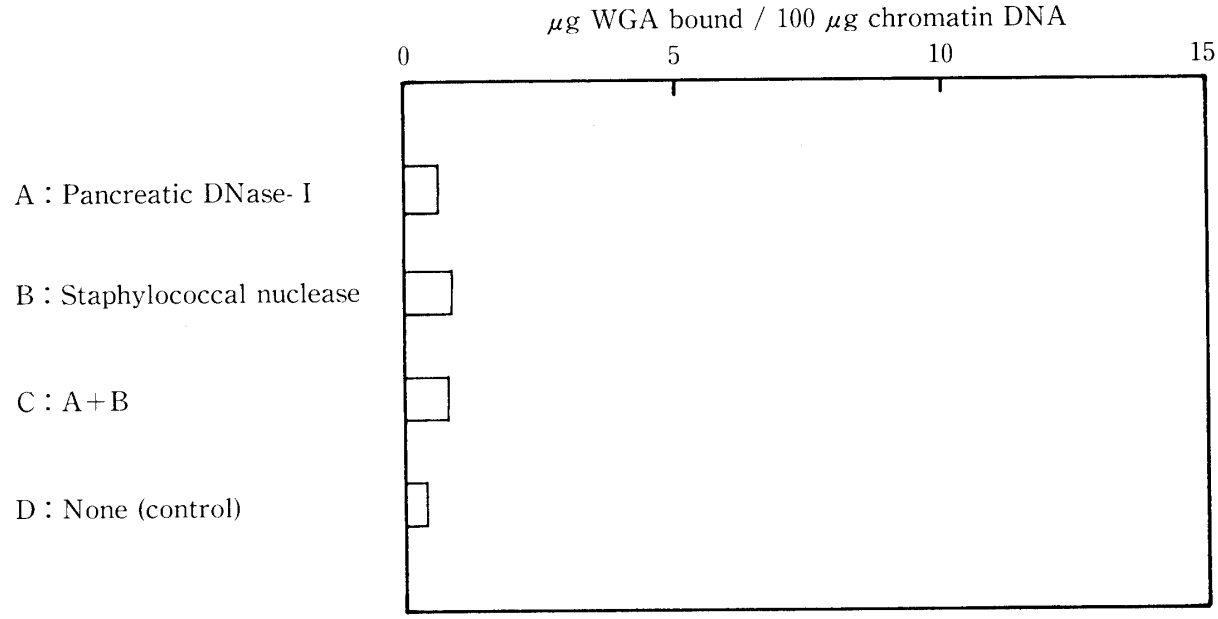


**Fig. 8.** Binding of WGA with sea urchin chromatin after treatment with pancreatic DNase-I and Staphylococcal nuclease. Reaction mixture (1.2ml) contained 100μg FITC-WGA, 60μg chromatin (as DNA), and 120 units of the enzyme. (*Clypeaster japonicus*)

*Extent of chromatin disintegration caused by pancreatic DNase-I and Staphylococcal nuclease*

The aim of digestion by the nucleotidases was not to disintegrate the chromatin exhaustively but to modify or loosen the chromatin structures to the extent of allowing discrimination of the regions of active sites from those of inactive ones in the chromatin.

In order to estimate the extent of digestion of chromatin, the amount of solubilized DNA was determined as an indication of disintegration during the course of enzyme digestion. Chromatin was incubated with the enzymes according to the protocol, and the amount of DNA remaining in the

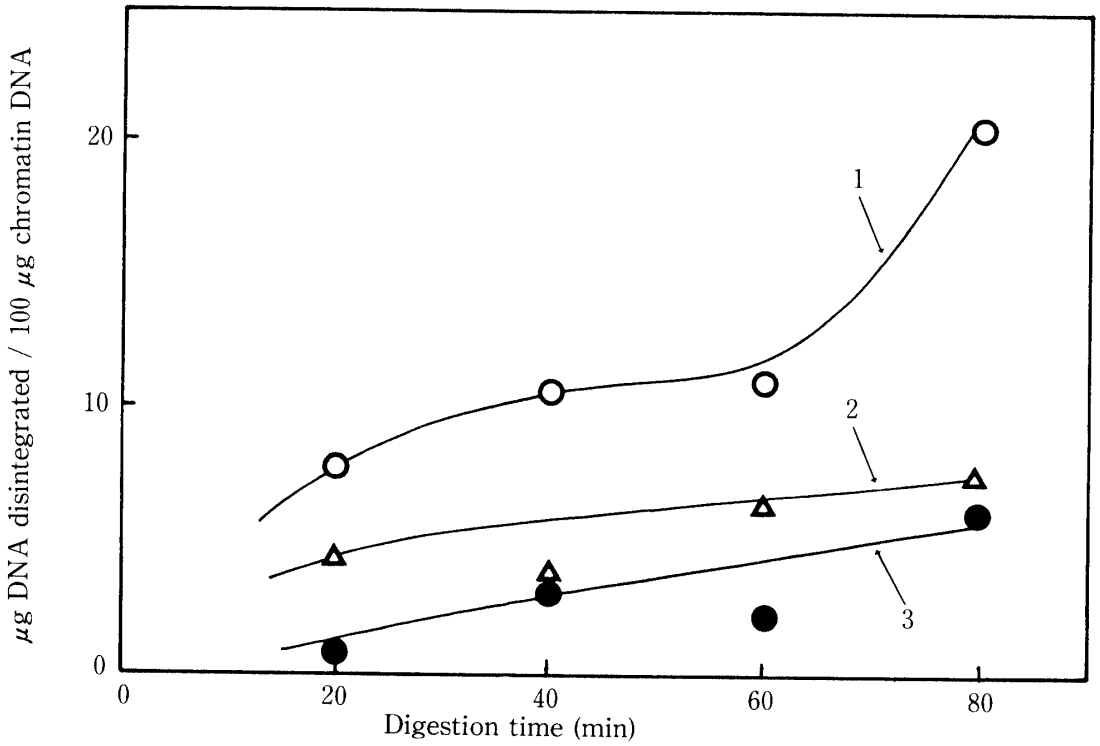


**Fig. 9.** Time course of chromatin disintegration during nucleotidase digestion as shown by leakage of DNA into the supernatant. 1(○): Pancreatic DNase-I; 2(△): Staphylococcal nuclease; 3(●): None (control) (*Clypeaster japonicus*)

supernatant after centrifugation was determined. The results were shown in Fig. 9. The extent of disintegration indicated by the leakage of DNA was observed to maintain a level of 10% of the total quantity of chromatin DNA. Since the incubation time with the two nucleotidases was limited to 30min in the digestion experiments stated above, the extent of disintegration was assumed to be minimal. The change in lectin binding ability observed in the native chromatin seemed to be due to the action of endogenous nuclease and the effect of sonication, as evidenced by the low level of lectin-binding in the non-sonicated chromatin (Fig. 10).

## DISCUSSION

It has been reported repeatedly that very diverse kinds of cell nuclei show affinity with lectins; for example, sea urchin chromatin with *Lens culinaris* lectin [21], polytene chromosome puffs in *Chironomus* salivary gland with Con A [22], high mobility group (HMG) nuclear proteins in calf thymus and mouse Friend cells with UEA-I [23], nuclei of rat liver with Con A [24-27].

In the present experiment it was demonstrated that the distribution of lectin-binding sites in the nucleus was confined to the dispersed areas of chromatin, as detected electron microscopically by means of the avidin-biotin complex technique. It was also demonstrated that considerable amounts of lectin-binding sites in chromatin became apparent by incubating the chromatin with pancreatic DNase-I which preferentially loosened the genetically active regions of chromatin, but the lectin binding sites were not exposed by the action of Staphylococcal nuclease, which showed no preference for the active regions.

Several points remain obscure in the present study. First, in the *in vitro* experiment the nuclear

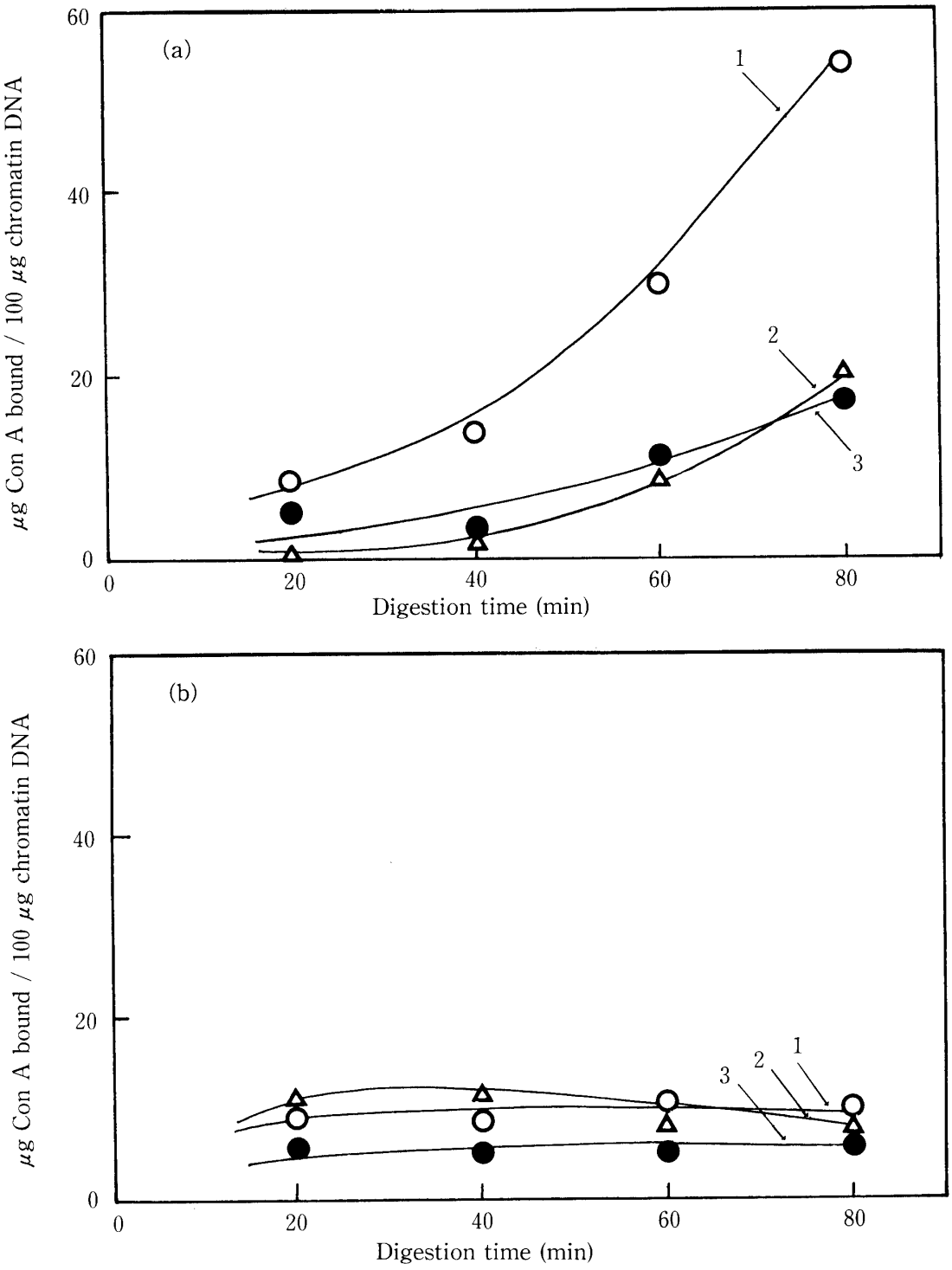


**Fig. 10.** Time course of Con A binding with sea urchin chromatin during nucleotidase digestion. 1(○): Pancreatic DNase-I; 2(△): Staphylococcal nuclease; 3(●): None (control). (a) Sonicated; (b) Not sonicated. (*Clypeaster japonicus*)

proteoglycans seem to be in a masked state in the native chromatin, in the sense that lectin was not accessible to the polysaccharide component until the chromatin was disintegrated by the action of pancreatic DNase-I, whereas in the electron microscopy study the polysaccharides were able to react with the lectin. The reason for the lectin reactability in the fixed specimen is still unknown. But it was frequently experienced in experiments examining the binding ability of FITC-labeled

lectins with the nuclear structures using permeabilized cells [10], that previous fixation with paraformaldehyde or glutaraldehyde was indispensable for lectin-binding. The fixation possibly serves as a kind of unmasking agent. The second point to be solved in future is, although the disintegration of chromatin remained at a minimal extent, 10% of chromatin DNA was lost during the period of digestion. This might be sufficient to modify the distribution and content of nuclear proteoglycans during this step of experiment.

Despite these points, the most reasonable conclusion drawn from the present experimental results would be the occurence of uneven distribution of proteoglycans within the chromation, presumably being more abundant in the euchromatic region and comparatively scarce in the heterochromatic region.

Concerning the function of nuclear proteoglycans there is no universal consensus. However, the following facts accumulated in our laboratory seems to suggest some role in the determination of the fate of embryonic cells through the modification of minute structures of the chromation. (1) Proteoglycans were transferred into the nucleus after synthesis in the cytoplasm, and bind with the chromatin as one of their constituents [2]. (2) The melting profile of the chromatin was shifted to a lower temperature by addition of exogenous proteoglycans, suggesting a loosening of chromatin structure, and thereby the RNA synthesis was augmented [2,4]. (3) The development of sea urchin embryo was arrested at the blastula stage when the synthesis of the proteoglycans was inhibited [6], and restored by administration of this substance [7]. (4) The newly synthesized proteoglycans bore a stage-specific character in the protein moiety of this substance, and sea urchin embryos ceased to develop by receiving excess proteoglycans by means of microinjection exactly at the stage when this proteoglycan was prepared [7].

Considering all these facts together, it is likely that the nuclear proteoplycans are candidates as regulating molecules in the chromatin, presumably at the level of the initial process in the activation mechanism of the genes in multi-steps, followed by the finer regulation molecules.

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