Differential Conservation of Histone 2A Variants between Mammals and Sea Urchins

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ABSTRACT The histone 2A proteins of the sea urchin *Strongylocentrotus purpuratus* are compared with those of the mouse. While the major H2As in these two organisms do not comigrate on two-dimensional gels, the sea urchin contains a protein that comigrates with the minor histone 2A variant H2A.Z from mammals. H2A.Z is of particular interest because its sequence homology with other H2As is quite low, and it is not phosphorylated as are other H2As. A comparison of the tryptic peptide patterns of several H2As from sea urchin blastulae and mouse L1210 cells shows that, while the patterns of the H2A.Zs differ greatly from the patterns of the other H2As, the patterns of the mouse and sea urchin H2A.Zs are very similar. Since the H2A.Zs have only one or two peptides in common with the other H2As, the conservation of their sequence indicates that H2A.Zs have evolved under somewhat different selective pressures from other H2As.

Unlike all the other sea urchin H2As whose syntheses either turn on or off during early development, H2A.Z seems to be synthesized continuously throughout this period.

This laboratory has previously characterized in mouse and other mammalian tissues two histone 2A-related proteins named H2A.X and H2A.Z (1, 2). H2A.Z in particular is quite different from H2A.1 both in modification pattern and in sequence although certain key peptides are conserved and, like H2A.1, it has ubiquitinated forms. In mouse, neither H2A.X nor H2A.Z comigrates with H2A.1 or H2A.2 in acetic acid/urea/Triton X-100, acetic acid/urea, or SDS gels; in the mammalian tissues and cells studied, each accounts for ~5–10% of the total H2A.

The histones of the sea urchin, the H2As in particular, have been well-characterized by sequence analysis (3, 4) and also by timing of synthesis during early development (5–11). Newrock et al. (5) and Childs et al. (6) described stage-specific H2A proteins and H2A mRNAs. Newrock et al. (7) showed that the synthesis of each of the H2A variants they observed is switched off or on at some point during early development. However, an H2A.Z was not recognized in these studies.

In this paper, we compare mouse H2As and some of the sea urchin H2As. Sea urchins contain a protein that comigrates with mouse H2A.Z and whose tryptic peptide pattern is almost identical with that of the mouse protein. Unlike the other sea urchin H2As, H2A.Z is synthesized continuously throughout early development.

MATERIALS AND METHODS

Embryo Cultures and Isotope Labeling

Undiluted semen and dejellied eggs of *Strongylocentrotus purpuratus* were obtained as described by Nishioka and McGwin (12). Insemination of 1% egg suspensions and culturing of embryos in artificial seawater were done according to Nishioka and Magagna (13). In those experiments involving embryos through the morula stage of development (pre-hatching), it was necessary to prevent the elevation and hardening of fertilization coats because they interfered with the nuclear isolation. The method of Epel (14) involving controlled treatment of unfertilized eggs with twice-crystallized trypsin (Sigma Chemical Co., St. Louis, MO) was employed. In those experiments involving embryos of the blastula and gastrula stages of development (post-hatching), the eggs were inseminated directly after the removal of the jelly coat. Embryos were maintained as a 1% suspension up to 20 h post-fertilization and as 0.5% suspension thereafter. Only cultures showing >95% normal fertilization and development were used. Embryos were labeled with [^{14}C]arginine or [^{14}C]lysine during the following periods of development: from fertilization to 2.5 h post-fertilization (four-cell stage), between 4 and 8 h post-fertilization (morula), between 14 and 18 h post-fertilization (hatching blastula), and between 32 and 36 h post-fertilization (gastrula) (13). The exact labeling conditions are given in the figure legends. All isotopes were purchased from ICN.

Cell Fractionation and Protein Extraction

Conditions for cell fractionation were those described in Wu and Wilt (15). Cultures of the different embryonic stages (50-ml aliquots) were removed at the end of the labeling period, washed three times in ice-cold 1.5 M dextrose by

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 MAY 1982 426-431
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centrifugation at 250 g, then suspended in 5 ml of 2 mM Mg acetate, 1 mM phenylmethylsulfonyl fluoride (PMSF) with several strokes in a glass homogenizer. After 10 min at 4°C, 5 ml of 2 M dextrose containing 2 mM Mg acetate, 1 mM PMSF was added and the suspension was homogenized with 20 strokes in a tight-fitting glass homogenizer (Kontes "B" type; Kontes Co., Vineland, NJ). The homogenate was then centrifuged at 3,000 *g* for 15 min in a Sorvall centrifuge (DuPont Co., Wilmington, Del.). The pellet was resuspended and carried through the same homogenization procedure one additional time. The pellet resulting from the second centrifugation was designated the nuclear fraction. The nuclear pellet was resuspended in 2 ml of 0.5 N HCl, 1 mM PMSF, 1% mercaptoethanol and extracted for at least 1 h at 4°C with frequent and vigorous shaking. The suspension was centrifuged in a microfuge for 5 min. The acid extracts were saved and processed as described in Bonner et al. (16).

Gel Electrophoresis

First-dimension acetic acid/urea/Triton X-100 (AUT) gels and second-dimension acetic acid/urea/hexadecyltrimethylammonium bromide (AUC) gels were prepared and run according to the methods described in the paper of Bonner et al. (16). Exact conditions are indicated in the figure legends.

Peptide Mapping

Proteins were isolated from gels, digested with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone trypsin, then electrophoresed on 50% acrylamide gels in 1 M acetic acid (1). 10,000-cpm [^{14}C]lysine- or [^{14}C]arginine-labeled protein was used for each analysis. The identity of the peptides in H2A.2 was determined using appropriate labels. For example, mouse H2A.1, H2A.2, H2A.X, and H2A.Z all contain the tryptic peptide ala-gly-leu-gln-phe-pro-val-gly-arg (R1) found in all sequenced H2As from animal as well as plant sources (17). As predicted by the sequence, this peptide is labeled in all four proteins with [^{14}C]leucine, phenylalanine, proline, valine, and arginine. It is not labeled with tyrosine, histidine, isoleucine or lysine (West, M. H. P., and W. M. Bonner, manuscript in preparation). A second peptide his-leu-gln-leu-ala-ile-arg is also found in all four proteins.

From these peptides and other model peptides it can be shown that on these peptide gels the log(R_f/number of cationic residues) is inversely proportional to the log(molecular weight) (Bonner, W. M., and M. H. P. West, manuscript in preparation).

Determination of Radioactivity

Autoradiograms and fluorograms were prepared by the methods of Bonner and Laskey (18) and Laskey and Mills (19). Gel slices were oxidized overnight at 37°C in 1 ml of H_2O_2/NH_4OH (95 parts/5 parts) in tightly capped scintillation vials. 15 ml of Aquasol scintillation liquid (New England Nuclear, Boston, MA) was added to each vial for counting.

RESULTS

Histones isolated from mouse L1210 cells were compared with histones isolated from sea urchin blastulae on two-dimensional gels (Fig. 1). The mouse pattern is repeated in the sea urchin blastula except that the major sea urchin proteins migrate a bit slower in the first dimension and somewhat faster in the second. Like their mouse counterparts, the sea urchin H2As, including the putative H2A.Z, and the H2Bs have ubiquitinated derivatives (20).

However, putative sea urchin H2A.Z seems to comigrate with mouse H2A.Z. That these two proteins comigrated in this two-dimensional gel system suggested that the sequences of these two proteins might have greater similarity than the sequences of the other mouse and sea urchin H2As. Therefore the sea urchin H2A proteins and the mouse H2As noted in Fig. 1 were isolated and their tryptic digests were compared on pH 3 peptide gels using methods previously developed in this laboratory (1, 2).

H2A Tryptic Peptide Patterns

The sequences of mammalian H2A.1 and H2A.2 (21, 22) and sea urchin total H2A are known and were compared by Isenberg (4). Fig. 2 shows peptide gels of mouse H2A.2 and

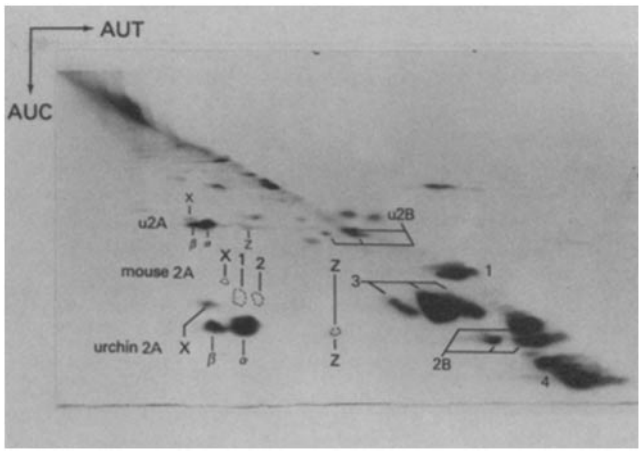

FIGURE 1 Comparison of histone from mouse L1210 cells with histone from sea urchin embryos. Sea urchin embryos were cultured as described in Materials and Methods, then labeled in seawater between 14 and 18 h postfertilization with [^{14}C]arginine at 2.5 μC/ml. The histones were extracted from nuclei, mixed with nonradioactive histones from L1210 cells, and then analyzed as described in Materials and Methods. The first-dimension AUT gel contained 8 M urea and 15% acrylamide and was run overnight at 7.5 mA. The second-dimension AUC gel contained 6 M urea and 16.5% acrylamide and was run overnight at 20 mA. The resultant fluorogram is displayed. The dotted areas denote the position of the 2A histones from L1210 cells as detected by stain.

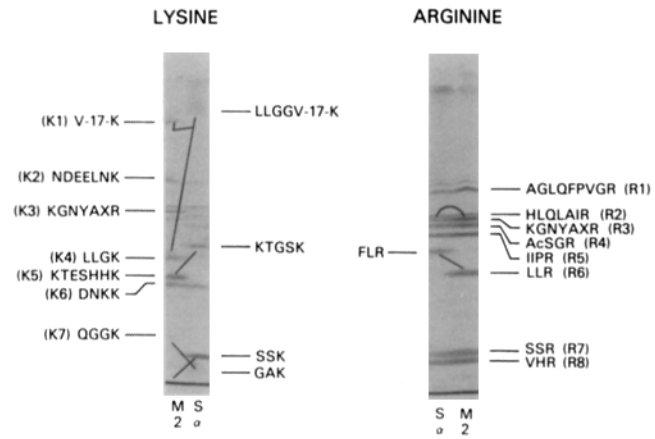

FIGURE 2 Tryptic peptide patterns of mouse H2A.2 and sea urchin H2A.α. Histones were isolated from mouse L1210 cells grown in the presence of [^{14}C]lysine (*K*) or [^{14}C]arginine (*R*) as described in West and Bonner (1). Embryos of *S. purpuratus* were grown in the presence of 5 μC/ml [^{14}C]lysine or [^{14}C]arginine from 14 to 18 h postfertilization. Histones were prepared from nuclei as described in Materials and Methods and were fractionated by gel electrophoresis (1). Particular histone species were extracted from the gels and digested with trypsin. The tryptic digests were analyzed on pH 3, 50% acrylamide gels (1). Gels were autoradiographed for 21 d. (*Left*) Autoradiograph of [^{14}C]lysine-labeled peptides K1–K7; (*right*) autoradiograph of [^{14}C]arginine-labeled peptides R1–R7; (*M2*) mouse H2A.2; (*S*α) *S. purpuratus* H2A.α. The identity of the mouse H2A.2 peptides has been determined by analyzing H2A.2 from L1210 cells grown in various labeled amino acids and comparing the labeling patterns of the bands with the patterns expected from the known sequence (21, 22). Differences between the H2A.2 and H2A.α patterns were correlated with sequence differences (4), using rules to relate the size and charge of a peptide to its mobility as described in Materials and Methods.

sea urchin H2A.α. For mouse H2A.2, the gel bands have been assigned to particular tryptic peptides and thereby to particular segments of the sequence by labeling the protein in vivo with specific amino acids (West, M. H. P., and W. M. Bonner, manuscript in preparation).

The arginine peptide patterns (Fig. 2, *right*) of the two proteins are very similar. The most obvious difference is the substitution of phe-leu-arg (FLR) for leu-leu-arg (LLR). There is another difference indicated in arginine peptides HLQLAIR and KGNYAXR, but the resolution in that region is not sufficient to clearly interpret the difference. Note that the slowest arginine peptide, ala-gly-leu-gln-phe-pro-val-gly-arg (AGLQFPVGR), present in both proteins, has been found in every H2A that has been sequenced from animals and plants (17).

The lysine peptide pattern (Fig. 2, *left*) shows more differences, most of which can be interpreted in terms of known sequence differences as indicated (4). Only two lysine peptides are conserved, asn-asp-glu-glu-leu-asn-lys (NDEELNK) and asp-asn-lys-lys (DNKK).

H2A.Z

The purpose of the preceding discussion was to set a basis for comparing the peptides of mouse and sea urchin H2A.Z with those of mouse H2A.2 and sea urchin H2A.α. These are shown in Fig. 3. It can be seen that, while mouse H2A.2 and sea urchin H2A.α have five arginine and two lysine peptides in common, mouse H2A.Z and putative sea urchin H2A.Z have seven arginine and eight lysine peptides in common. The H2A.Zs are clearly more conserved than the major H2A variants in each of these organisms. It should also be pointed out that there are no lysine peptides found in both the H2A.Z and the major H2A variants and that only one arginine peptide (band 1, Fig. 3, *right*) is found in both. (Another arginine peptide [band 2, Fig. 3, *right*] is found in mouse H2A.Z and H2A.2 but seems to be altered slightly in sea urchin H2A.α.) The data in Fig. 3 show that mouse H2A.Z and the putative sea urchin H2A.Z are very similar proteins but at the same time very different from the major H2As in both these organisms.

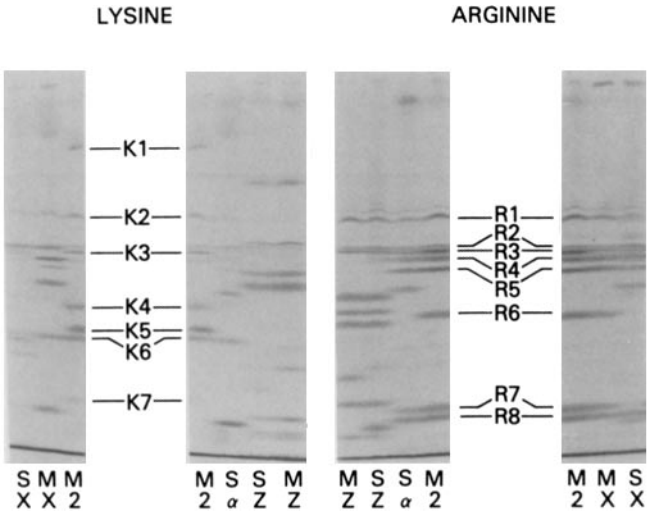

FIGURE 3 Tryptic peptide patterns of mouse and sea urchin H2A.Z and H2A.X compared with those of mouse H2A.2 and sea urchin H2A.α. Methods are as described in Fig. 2. (*Left*) 21-d autoradiograph of [^{14}C]lysine-labeled (*K*) peptides; (*right*) 21-d autoradiograph of [^{14}C]arginine-labeled (*R*) peptides. (*M2*) Mouse H2A.2; (*MX*) mouse H2A.X; (*MZ*) mouse H2A.Z; (*S*α) *S. purpuratus* H2A.α; (*SX*) *S. purpuratus* H2A.X; (*SZ*) *S. purpuratus* H2A.Z.

In the case of mouse H2A.2, the peptides separated on these gels are known to account for all the H2A tryptic peptides with two exceptions. The first exception is a large acidic peptide containing 29 amino acids which does not migrate into pH 3 peptide gels because of its low net charge and insolubility. This peptide, which can be analyzed on pH 9 peptide gels (2), was also found in sea urchin H2A.α (data not presented). However, H2A.Z in mouse and sea urchin both lack this peptide, and in fact the H2A.Zs have no arginine peptides on pH 9 gels. Since the H2A.Zs have several more bands on the pH 3 gels (Fig. 3), it is possible that, instead of the 29 amino acid peptide, the H2A.Zs have smaller peptides in this region that migrate on pH 3 gels.

The second exception is the tryptic dipeptides that migrate with the buffer discontinuity and are unresolved. Mouse H2A has eight of these, all but one of which are located near the NH_2 terminal or COOH-terminal ends. Although certain modifications to the pH 3 peptide gel allow many of the dipeptides to be separated, we have not compared dipeptides in this study.

H2A.X

The sea urchin protein labeled H2A.X in Fig. 1 does not comigrate with mouse H2A.X but is found in a similar position relative to the major H2As of each organism.

Fig. 3 also shows a comparison of the peptides of mouse H2A.X and sea urchin H2A.X. The two proteins differ at several sites and each contains several peptides in common with the respective H2A.2. For example, sea urchin H2A.X has the peptide phe-leu-arg (see Fig. 2) in common with sea urchin H2A.2, while mouse H2A.X has the peptide leu-leu-arg (R6) in common with its respective H2A.2. While only one peptide could be shown to be common to H2A.Z and H2A.α, the H2A.Xs have four arginine and two lysine peptides in common with the major H2As. Both H2A.Xs also contain a 29 amino acid peptide found on pH 9 gels.

These data substantiate the conclusion originally reached in mouse (1) that the H2A.Xs have much more sequence homology with the major H2As than does H2A.Z. In addition the experiments show that the H2A.Xs and major H2A have similar amounts of sequence variation between mouse and sea urchin, but that the H2A.Z sequences are much more conserved.

Developmental Studies

Several laboratories have investigated and described the process of histone switching during sea urchin development both at the protein level and at the mRNA level (5–11). The conclusion of these studies is that the H2As being synthesized in the gastrula are not the same ones being synthesized in early cleavage or in the morula.

However, H2A.Z was not recognized as an H2A at the time of these studies. In light of the high degree of sequence conservation between mouse and sea urchin H2A.Z, we decided to reinvestigate the developmental controls in H2A synthesis in sea urchin with specific reference to H2A.Z.

Our results (Fig. 4 and Table I) agree with those of earlier authors concerning the H2As that they studied, and we have tried to maintain their terminology (5, 6). Cs2A accounts for most of the 2A synthesis at the two-cell stage, but its share rapidly drops. H2A.α accounts for the vast majority of the

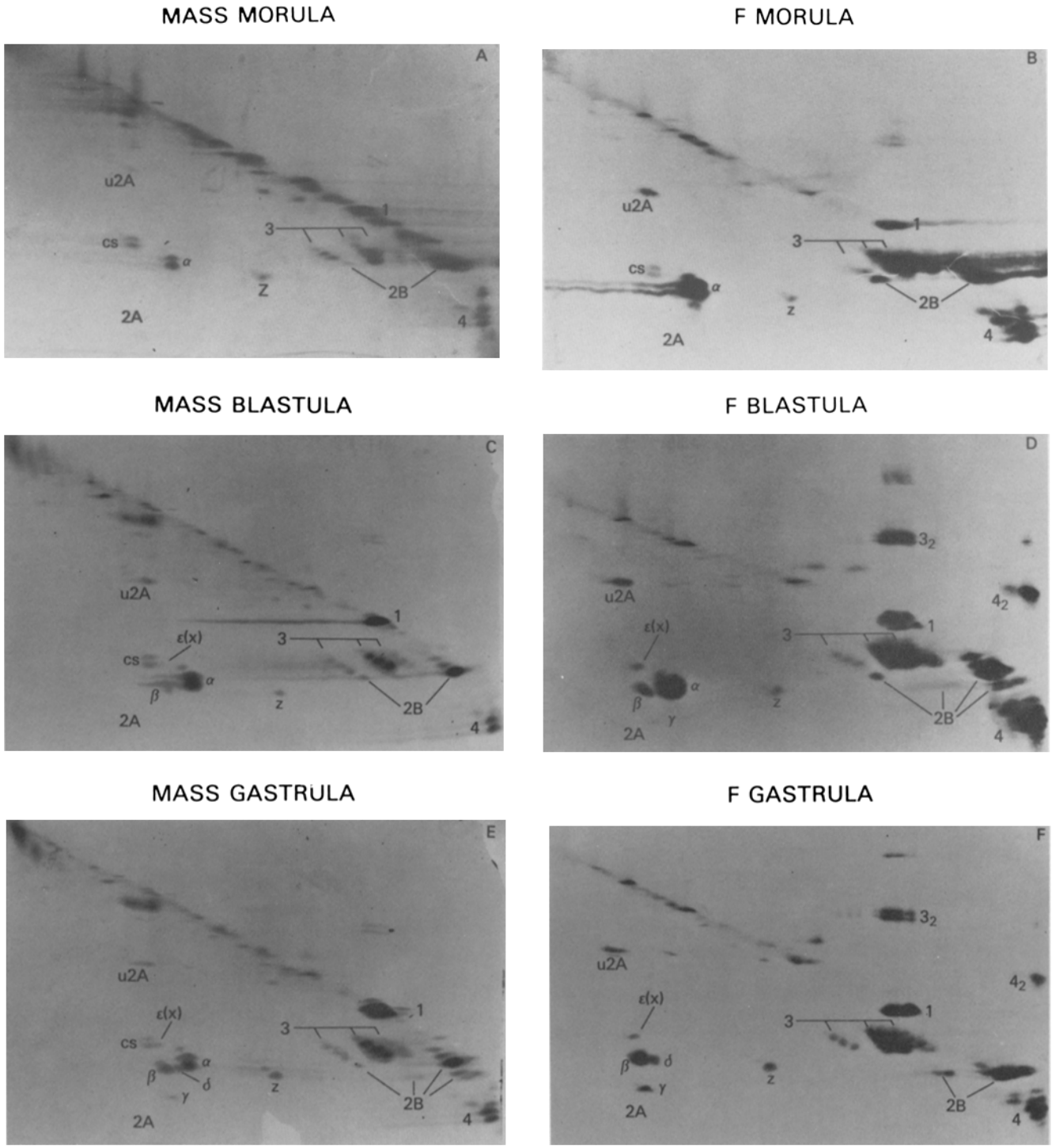

FIGURE 4 Histone switching during embryo genesis. Sea urchin embryos at various stages of development were labeled in sea water with [^{14}C]arginine at 2.5 μC/ml as described in Materials and Methods. The histones were extracted from the nuclear pellet and analyzed on two-dimensional gels. The first-dimension AUT gel contained 8 M urea, 10% acrylamide; the second-dimension AUC gel contained 6 M urea, 16.5% acrylamide. The remaining conditions are those described in Fig. 1. H2A.CS and H2A.α show a doublet on these gels. This is usual for methionine containing H2As (16), and these two sea urchin H2As are known to contain methionine (7, 10). (*A* and *B*) Morula, (*C* and *D*) blastula, (*E* and *F*) gastrula. (*A*, *C*, and *E*) mass patterns, (*B*, *D*, and *F*) fluorographs.

synthesis in the morula and blastula, but its synthesis is turned off in the gastrula. Synthesis of the various late forms are detectable in the blastula, with the H2A.β accounting for about half the synthesis in the gastrula.

In contrast, H2A.Z is synthesized at all four stages studied (Table I). It accounts for ~0.10 of H2A synthesis at the two-cell stage; its share drops to 0.01 and 0.03 in the morula and blastula, respectively, and rises to 0.18 in the gastrula. However, the nuclei are multiplying rapidly during early development; therefore total H2A synthesis must also be greatly increasing up to the blastula stage. Assuming that in sea urchin the rate of histone synthesis is proportional to the rate of DNA synthesis which in turn should be proportional to the increase in cell number (ΔC) during the labeling period, one can compare

TABLE I

Histone 2A Switching during Embryogenesis

	Cleavage		Morula		Blastula		Gastrula	
Time, *h*	0–2.5		4–8		14–18		32–36	
Cells per embryo	4		40		300		600	
Δ Cells per embryo	3		34		130		50	
Variant	V/T	(V/T)ΔC	V/T	(V/T)ΔC	V/T	(V/T)ΔC	V/T	(V/T)ΔC
CS	0.69	2.1	0.02	0.68		—		—
α	0.21	0.63	0.97	33.0	0.849	110.4		—
ε(X)		—		—	0.020	2.6	0.03	1.50
β		—		—	0.099	12.9	0.49	24.50
γ		—		—	0.005	0.65	0.10	5.00
δ		***		***		***	0.20	10.00
Z	0.10	0.30	.01	0.34	0.027	3.5	0.18	9.00

Histone 2A switching during embryogenesis. The 2A spots were cut out from the gels shown in Fig. 5. A gel of early cleavage embryos, showing the same H2As as the gel of morula, was not displayed in Fig. 5 but was included in this quantitation. Spots were cut out from the dried gel, digested, and their radioactivity was quantitated as described in Materials and Methods. The radioactivity in each variant (*V*) is divided by the radioactivity in total (*T*) H2A labeled at that time. The fractional synthesis (*V*/*T*) is independent of the specific activity of the amino acid pools. In the second column [(*V*/*T*)ΔC], the fractional synthesis (*V*/*T*) of each variant is multiplied by the increase in the number of cells per embryo (ΔC) during the labeling period. Since this increase should be directly proportional to the rate of DNA synthesis and hence to total H2A synthesis, one is able to compare the rates of synthesis of each variant at different stages. Therefore although the fractional synthesis of H2A.X (*V*/*T*) falls from 0.10 to 0.01 between early cleavage and morula, the rate of H2A.Z synthesis per embryo does not change very much. Cells per embryo and Δ cells per embryo were calculated from the labeling times according to Hinegardner (26).

The gels in Fig. 5 all show ubiquitinated H2As. However, the fraction of H2A in the ubiquitinated form was not included in this table, because ubiquitin on the H2A is in rapid equilibrium with free newly synthesized ubiquitin (27), which would become labeled during the pulse. Because of this, H2As whose syntheses are totally shut down will have label in the ubiquitin adduct. In any case the amount of H2A in the ubiquitin form does not significantly alter the results in this table. —, Not detectable; ***, not determined due to insufficient separation.

variant synthesis during development. Table I shows that H2A.Z synthesis remains fairly constant through morula and then greatly increases in the blastula and gastrula. These synthetic rates reflect only the histone found in isolated nuclei; a measurement of total cellular histone may yield somewhat different rates. Nevertheless these results suggest that the fall in the fractional synthesis of H2A.Z between cleavage and morula is not due to a decrease in the rate of H2A.Z synthesis but rather to a very large increase in the rate of H2A.α synthesis.

The timing of H2A.X synthesis during development suggests that it is a late variant appearing first during the blastula, probably corresponding to H2A.ε (6). This result helps to substantiate our earlier conclusion, based on its homology with respect to H2A.α and its evolutionary variability, that H2A.ε(X) is probably functionally similar to the major H2As.

DISCUSSION

Sea urchin embryos contain a protein that comigrates in several gel electrophoresis systems with mouse H2A.Z. The peptide maps indicate that the mouse and sea urchin proteins are very similar and that both are very different from the other H2As. H2A.Z is synthesized continuously throughout early sea urchin development and, in this respect also, seems to be different from other sea urchin H2As.

Previous characterization of mouse H2A.Z showed that it contains the arginine tryptic peptide found so far in all sequenced H2As from animals as well as plants, and a significant proportion of H2A.Z is modified with ubiquitin. Otherwise, it differs considerably in modification and in sequence from the other H2As and also, as shown here, in the conservation of that sequence. An H2A.Z has been found in every tissue and cell type we have studied, usually accounting for between 1 and 10% of the total H2A.

Proteins whose migration is similar to that of mouse H2A.Z have been reported in *Tetrahymena* (23), *Drosophila* (24), and chicken (25). Both the *Tetrahymena* protein hvl and *Drosophila* protein D2 were shown to have several tryptic peptides that comigrated with H2A peptides, even though there were considerable differences. These findings further suggest that an H2A.Z-like protein may be a universal component of eukaryotic chromatin.

Mouse H2A.Z is not phosphorylated and has three modified forms, whereas the other mouse H2As including H2A.X are phosphorylated and have two modified forms. We have not checked specifically whether or not sea urchin H2A.Z is phosphorylated, but its modification seems to be similar to that of the mouse protein.

On the other hand, mouse H2A.X is much more similar to mouse H2A.1 or H2A.2 than is mouse H2A.Z. H2A.X is modified with phosphate and acetate, as are H2A.1 and H2A.2 (2) and has large regions of identical sequence (West, M. H. P., and W. M. Bonner, manuscript in preparation). The data reported here indicate that sea urchin H2A.ε(X) also bears a similar relationship to sea urchin H2A.α. Sea urchin H2A.ε(X) has the tripeptide phe-leu-arg found in sea urchin H2A.α and H2A.β, whereas mouse H2A.X has the tripeptide leu-leu-arg found in mammals. Therefore, this evidence suggests that these two H2As reflect the sequence variation allowed for functional H2As, although functional differentiation has not been ruled out.

In contrast to the H2A.Xs, sea urchin and mouse H2A.Z have similar sequences, yet these sequences are quite different from the major H2As of each species. This degree of conservation is more like the highly conserved H4s and H3s rather than the other H2As which have multiple differences between sea urchin and mouse. The synthesis of H2A.Z throughout early development is also more characteristic of the H4s and H3s than the other H2As or H2Bs. These results indicate that

H2A.Z is a very widely, if not universally, distributed protein in eukaryotes whose function maintains rather strict selective pressure on its sequence. They further suggest that H2A.Z has a basic cellular function at least partially differentiated from the functions of the other H2As.

We wish to thank Professor Daniel Mazia for the use of his facilities at the University of California, Berkeley, while D. Nishioka was a visiting investigator (summer, 1980). We also thank Madie Tyler for typing this manuscript and Irene Clark for maintaining L1210 cells.

This work was supported in part by National Science Foundation grant 7923487 to D. Nishioka.

Received for publication 11 August 1981, and in revised form 30 November 1981.

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