Vandekerckhove, J., & Weber, K. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1106-1110.
Vandekerckhove, J., & Weber, K. (1978b) *Eur. J. Biochem. 90*, 451-462.
Vandekerckhove, J., & Weber, K. (1978c) *J. Mol. Biol. 126*, 738-802.
Vandekerckhove, J., & Weber, K. (1978d) *Nature (London) 276*, 720-721.

Embryonal Histone H1 Subtypes of the Sea Urchin *Strongylocentrotus purpuratus*: Purification, Characterization, and Immunological Comparison with H1 Subtypes of the Adult†

John R. Pehrson* and Leonard H. Cohen

ABSTRACT: The four H1 subtypes utilized during embryonic development of *Strongylocentrotus purpuratus* were purified. Their amino acid compositions and immunological reactivities show that they have different primary structures. Antisera against them were used to identify the H1 subtypes present in adult tissues. These experiments revealed that adult tissues do not contain detectable levels of the two H1 subtypes expressed in the earliest embryonic stages (H1cs and H1α) but do contain the two subtypes expressed from the blastula stage on (H1β and H1γ). In addition, all adult tissues examined contain H1λ, a subtype of unusually low apparent molecular weight, which is not found in the embryo prior to the feeding larval stage and is not closely related immunologically to any of the embryonal H1 subtypes.

Histone H1 exhibits the greatest variation in primary structure of the five major histone classes (Kinkade & Cole, 1966; Kinkade, 1969; Rall & Cole, 1971). Most organisms contain more than one H1 protein (termed H1 subtypes), and in mammalian cells, the subtypes present and their relative proportions depend on the cell type (Bustin & Cole, 1968; Kinkade, 1969; Seyedin & Kistler, 1980). Since H1 is involved in higher order chromatin structure (Finch & Klug, 1976; Thoma & Koller, 1977; Thoma et al., 1979; Butler & Thomas, 1980), it seems possible that different H1 subtypes may give rise to different higher order structures and in so doing affect some coarse regulation of genetic expression. This notion is supported by the observations that the proportions and kinds of H1 subtypes synthesized can be changed by hormonal stimulation (Hohmann & Cole, 1971) and by inhibition of cell division (Panyim & Chalkley, 1969a; Pehrson & Cole, 1980, 1982; Lennox & Cohen, 1983) and that the different subtypes are phosphorylated to different degrees and on different schedules during the cell cycle (Ajiro et al., 1981; Lennox et al., 1982).

The sea urchin embryo undergoes dramatic changes in the expression of H1 subtypes during its development (Hill et al., 1971; Ruderman & Gross, 1974; Newrock et al., 1978). H1cs, the H1 subtype that is stored in the egg (Newrock et al., 1978; Salik et al., 1981), is the predominant subtype present during the first five cell divisions; these cell divisions occur about once an hour. Next, H1α appears and by the blastula stage is the major H1 subtype; during this time, the rate of cell division slows significantly. Two other H1 subtypes, H1β and H1γ, appear in the chromatin during the blastula stage, and by gastrulation, they are the only H1 subtypes being made by the embryo; during this period, cell proliferation continues to slow, and many noncycling cells appear for the first time (L. H. Cohen and G. McFadden, unpublished results). A fifth H1 subtype, H1λ, first appears in the late larva and is an abundant subtype in adult tissues (L. H. Cohen, K. Newrock, and R. Hinegardner, unpublished results). A gene coding for *Strongylocentrotus purpuratus* H1α has been cloned and sequenced (Levy et al., 1982), and three *Parechinus angulosus* H1 subtypes resembling H1α, H1β, and H1γ in the timing of their expression during development have been purified and partially sequenced (De Groot et al., 1983). In this paper, we report the purification of the four *S. purpuratus* embryonal H1 subtypes and some chemical and immunological comparisons of these proteins. We have succeeded in preparing antibodies that are monospecific against H1cs and H1α and have used these together with unabsorbed antisera to investigate whether these subtypes are present in the tissues of the adult.

Materials and Methods

Nuclear Isolation and Histone Extraction. Cultivation of embryos and isolation of nuclei from embryos were carried out as described previously (Cohen et al., 1975). Adult tissues were obtained from male urchins to avoid contamination by eggs or oocytes containing H1cs. Intestines were dissected from the animal and washed several times with ice-cold seawater to remove digestive juices and food. To obtain tube feet, urchins were rapidly removed from glass beakers, leaving many tube feet attached to the beaker. Coelomocytes were obtained by first injecting the urchin with 2 mL of 10% ethylenediaminetetraacetic acid (EDTA),[1] pH 7.0, to inhibit clotting. Aristotle's lantern was then cut away, and the coelomic fluid (usually ~10 mL per urchin) was drained into a chilled beaker containing 2 mL of EDTA per urchin. The coelomic fluid was filtered through cheesecloth, and an equal volume of 0.8 M dextrose was added. Coelomocytes were spun down at 1000*g* for 10 min. The following procedures were all performed at 0-4 °C. The tissues were homogenized with a Dounce hom-

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received June 5, 1984.* This work was supported by Grants CA06927, CA24019, and RR05539 from the National Institutes of Health and an appropriation from the Commonwealth of Pennsylvania. This work was also supported by Grant CA-09035 from NIH Training Grant 1801.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-6761$01.50/0

ogenizer in buffer A (0.34 M sucrose, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA, and 15 mM Tris, pH 7.4) (Hewish & Burgoyne, 1973) supplemented with 1 mM phenylmethanesulfonyl fluoride. The homogenate was filtered through cheesecloth and a 41-μm Nitex screen (the filtration step was omitted with coelomocytes). The nuclei were pelleted by centrifugation at 1000g for 6 min. They were suspended in buffer A containing 0.1% Triton X-100, left on ice for 10 min, and centrifuged. The nuclei were washed once more with buffer A without Triton X-100 before histones were extracted. Tube feet were difficult to homogenize and gave a poor yield of nuclei. When coelomocytes are homogenized, the solution can become viscous and make it difficult to pellet the nuclei; this viscosity diminished after a few minutes at 0 °C. Isolated nuclei were extracted twice with 0.4 N H_2SO_4, the extracts were combined, and perchloric acid was added to a final concentration of 0.74 M to precipitate core histones and most non-histone proteins. The precipitated core histones were removed by centrifugation, and then H1 was precipitated by adding trichloroacetic acid to 20%. The trichloroacetic acid precipitate was centrifuged, washed twice with ethanol, and dried.

Purification of H1 Subtypes. Approximately 5 mg of H1 from 4-day embryos was fractionated at room temperature on a column (0.9 × 20 cm) of Bio-Rex 70, (Bio-Rad) 100–200 mesh. The column was developed with a linear gradient (600 mL) of 7–14% (w/v) guanidinium chloride in 0.1 M sodium phosphate, pH 6.5, at a flow rate of 5.6 mL/h. Pooled fractions were desalted on a Sephadex G-25 column equilibrated with 0.01 N HCl and lyophilized. H1cs was purified by preparative gel electrophoresis (Newrock et al., 1978) on a 6-cm-long 12% polyacrylamide gel containing 8 M urea and 5% acetic acid (Panyim & Chalkley, 1969b). The histones were collected in 5% acetic acid, and samples of selected fractions were lyophilized and electrophoresed in SDS–polyacrylamide gels. The H1cs-containing fractions were pooled, desalted on a Sephadex G-25 column equilibrated with 5% acetic acid, and lyophilized. Purified H1 proteins were hydrolyzed in 6 M HCl containing 0.1% phenol, at 110 °C, for 20 h. The amino acids were separated and quantified on a Durrum amino acid analyzer.

Immunological Procedures. Rabbits were immunized with an H1–RNA complex as described (Stollar & Ward, 1970). Cross-reacting antibodies were removed by passage of antisera through columns containing purified H1 subtypes immobilized on Affi-10 (Bio-Rad). Enzyme-linked immunoassays were performed in microtiter wells essentially as described (Allan et al., 1982) except Triton X-100 was used instead of Tween and bovine serum albumin was used instead of horse serum.

Electrophoresis was performed in 18% polyacrylamide gels containing SDS (Laemmli, 1970). For immunological identification of the H1 subtypes, we have found that the electrophoretic transfer from SDS gels to nitrocellulose paper was greatly improved by removal of the SDS prior to electrophoresis of the proteins as anions in acetic acid. SDS was removed from the gel by soaking it 3 times (2, 18, and 2 h) in 25% 2-propanol–10% acetic acid. The 2-propanol was removed by soaking the gel twice in 5% acetic acid (1 h each time), and then the histones were electrophoretically transferred to nitrocellulose paper in 5% acetic acid. The nitrocellulose paper was soaked twice in phosphate-buffered saline to removed excess acetic acid and then treated overnight with phosphate-buffered saline containing 2 mg/mL bovine serum albumin. The paper was incubated in H1 antiserum (diluted 1:150), and peroxidase-conjugated goat anti-rabbit antiserum

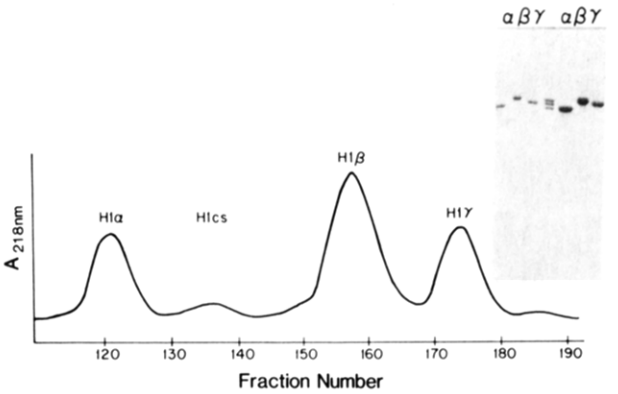

FIGURE 1: Chromatographic separation of *S. purpuratus* embryonal H1 subtypes. Perchloric acid soluble nuclear proteins isolated from 4-day embryos were chromatographed on Bio-Rex 70. (Insert) SDS–polyacrylamide gel electrophoresis of purified H1 subtypes. The middle lane is the unfractionated mixture of H1 subtypes.

(Miles) was used at a 1:500 dilution as the second antibody. The incubation periods and washing procedures were the same as those used for the microtiter well procedure. The bands that reacted with the antisera were visualized by addition of H_2O_2 and 4-chloronaphthol (Hawkes, 1982).

Results

The perchloric acid soluble proteins extracted from purified sea urchin embryo nuclei were chromatographed on Bio-Rex 70 (Figure 1). The peaks containing H1α, H1β, and H1γ were free of contaminants as judged by electrophoresis in SDS–polyacrylamide gels (see inset of Figure 1). H1cs was resolved from the other subtypes but was not efficiently recovered from the column. We attempted to purify H1cs by gel filtration chromatography since its molecular weight, as judged by electrophoresis in SDS gels, is much larger than those of the other sea urchin H1 subtypes. However, while gel filtration on Bio-Gel P-100 gave partial resolution of H1α, H1β, and H1γ from one another, H1cs was eluted together with H1γ (results not shown). H1cs was therefore purified by preparative electrophoresis in a polyacrylamide gel containing acetic acid and urea (Newrock et al., 1978).

Amino acid analyses of the purified proteins, along with the amino acid composition of a sea urchin H1 deduced from a cloned gene (Levy et al., 1982), are shown in Table I. The amino acid compositions have characteristics of H1 proteins of other organisms, i.e., a high content of lysine, alanine, and proline and a paucity of hydrophobic amino acids. They also establish that the four subtypes have different primary structures. H1α closely resembles the H1 gene cloned in pSp2 (Levy et al., 1982) and, as seen from the underlined numbers, differs from H1β, H1γ, and H1cs.

Antisera were raised in rabbits against each of the four sea urchin embryo H1 subtypes, and enzyme-linked immunoassays were used to measure the reaction of each antiserum with purified H1 proteins (Table II). The results show that the four sea urchin embryo H1 subtypes are immunologically distinct. The greatest similarity is between H1β and H1γ, which react equally well with the antisera raised against them, but which are readily distinguished by anti-H1α and anti-H1cs. The lower half of Table II shows that absorption of antisera with purified H1 proteins yields specific antisera. This was also apparent in Western transfers. Embryonal H1 proteins were electrophoresed in SDS gels, transferred to nitrocellulose, and probed with the absorbed antisera (Figure 2, lanes b–d). These results show that the absorbed antisera identified H1 subtypes with a high degree of specificity. H1γ can be dis-

Table I: Amino Acid Compositions of *S. purpuratus* Embryonal H1 Subtypes[a]

	H1cs[b]	H1α	H1β	H1γ	pSp2[c] gene
Asp	6.8	3.6	2.0	1.6	3.5
Thr	4.7	5.9	2.9*	3.8*	6.4
Ser	7.9	4.8	4.1	4.1	5.9
Glu	6.8	10.4	7.0*	5.7*	10.9
Pro	10.2	6.1	5.0*	6.9*	6.9
Gly	4.6	3.3	4.4	5.0	4.5
Ala	13.7	19.5	25.8	25.3	16.3
Val	5.1	4.5	3.7*	5.3*	4.5
Met	1.7	1.0	0.5*	1.0*	1.0
Ile	4.0	1.9	3.2*	1.9*	2.0
Leu	3.5	4.7	3.4	3.0	4.0
Tyr	1.6	0.5	0.9	1.0	0.5
Phe	1.1	1.5	1.0	1.0	1.5
His	1.1	0.6	0.5	0.6	0.5
Lys	22.3	29.2	32.4	30.4	29.2
Arg	4.9	2.2	3.1	3.3	2.5

[a] Values are expressed as moles per 100 mol of total amino acid recovered. No corrections were made for the oxidation of serine and threonine. Underscored values are those that differ from the values obtained for H1α. Asterisks indicate differences between H1β and H1γ. [b] Data from Newrock et al. (1978). [c] Values calculated from data of Levy et al. (1982).

tinguished from H1β by its reaction with unabsorbed anti-H1cs (Figure 2, lane e).

We compared the H1 subtypes of three adult tissues (coelomocytes, tube feet, and intestines) with those of the embryo by electrophoresis and immunological reactivity. SDS gel electrophoresis revealed that these tissues contain three major forms of H1, as shown for intestine H1 in Figure 3, lane b; all three tissues had very similar H1 patterns. Two of the proteins comigrate with embryonic H1β and H1γ while the third moves faster than H1α (Figure 3, compare lanes a and b). This third H1 is not present in the embryo of *S. purpuratus* but appears at a later larval stage of development and has been termed H1λ (L. H. Cohen, K. Newrock, and R. Hinegardner, unpublished results). When H1 subtypes of adult tissues were transferred from SDS gels to nitrocellulose paper and probed with H1 antisera, neither H1cs nor H1α was detected (Figure 3, lanes c and e). These studies also revealed that the putative H1β and H1γ of adult tissues are like H1β and H1γ of the embryo in two respects: (a) both reacted strongly with anti-H1γ (absorbed against H1α) (Figure 3, lane d); and (b) H1γ reacted much better with anti-H1cs than did H1β (Figure 3, lane f). Thus, adult H1β and H1γ are almost certainly the same two proteins as those in the embryo. H1λ shows very little reaction with any of the absorbed antisera (Figure 3, lanes c–e), but it does have some reaction with unabsorbed anti-H1α and anti-H1γ (Figure 3, lanes g and h).

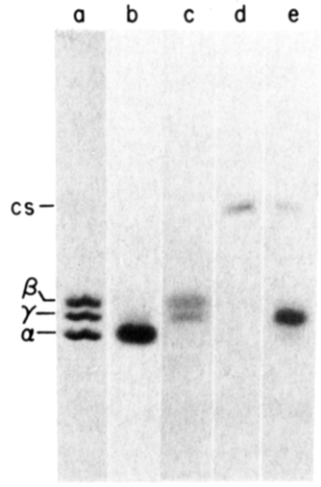

FIGURE 2: Immunological identification of embryonal H1 subtypes. The H1 subtypes of 3-day embryos were electrophoresed in an SDS–polyacrylamide gel, transferred to nitrocellulose paper, and reacted with H1 antisera raised in rabbits. Bound H1 antibodies were reacted with peroxidase-conjugated goat anti-rabbit antiserum, followed by the addition of 4-chloronaphthol and H_2O_2 to visualize the complex. Lane a, Coomassie blue stained gel. Lanes b–e, H1 proteins transferred to nitrocellulose and reacted with (b) anti-H1α absorbed against H1γ, (c) anti-H1γ absorbed against H1α, (d) anti-H1cs absorbed against H1α, H1β, and H1γ, and (e) unabsorbed anti-H1cs.

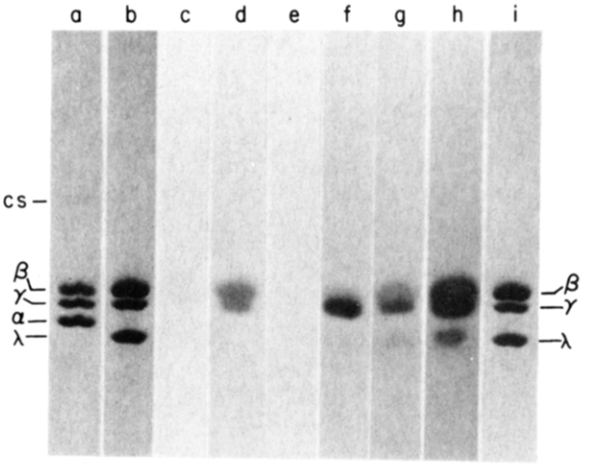

FIGURE 3: Reaction of H1 subtypes of adult intestine with H1 antisera. The H1 subtypes of adult intestines were electrophoresed, transferred to nitrocellulose, and reacted with antisera as described in Figure 2. Lane a, gel of embryonal H1 subtypes stained with Coomassie blue; lanes b and i, stained gel of adult intestine H1 subtypes; lanes c–h, adult intestine H1 subtypes reacted with (c) anti-H1α absorbed against H1γ (d) anti-H1γ absorbed against H1α, (e) anti-H1cs absorbed against H1α, H1β, and H1γ, (f) unabsorbed anti-H1cs, (g) unabsorbed anti-H1α, and (h) unabsorbed anti-H1γ.

Discussion

The H1 subtypes expressed during the embryonic development of the sea urchin have some unusual characteristics. H1cs, the H1 utilized for chromatin formation at the earliest embryonic stages, exhibits an unusually large size as measured

Table II: Reaction of H1 Antisera with Purified H1 Proteins:[a] Percent of Reaction of Homologous Antiserum

	H1α	H1β	H1γ	H1cs	H1, mouse[b]	H1°, mouse[b]	H1, *Drosophila*[c]
sera							
anti-H1α	100	19	40	10	4	2	9
anti-H1β	29	100	100	9	9	3	7
anti-H1γ	42	100	100	11	5	5	20
anti-H1cs	5	6	45	100	<2	2	2
absorbed sera[d]							
anti-H1α (γ)	100	7	5	20			
anti-H1γ (α)	4	100	100	8			
anti-H1cs (αβγ)	<2	<2	<2	100			

[a] Antisera reactions were measured by enzyme-linked immunoassays (see Materials and Methods). [b] H1° was separated from the other H1 subtypes of mouse kidney by use of Bio-Gel P-100 (Pehrson & Cole, 1980). [c] *Drosophila* H1 was a generous gift of Dr. C. R. Alfageme. [d] The H1 subtypes used for absorption are in parentheses.

by SDS gel electrophoresis; this, together with its somewhat low lysine and alanine content, cast some uncertainty on the identification of this protein as an H1 histone, which was based originally on the fact that it is the only protein soluble in perchloric acid that is present in an appropriate amount in nuclei of the cleavage-stage embryo (Newrock et al., 1978). However, the immunological evidence gives strong support to this identification. Thus, H1cs reacted with all of the antisera raised against other sea urchin H1 subtypes at least as well as did H1 from other species (the only exception being the reaction of *Drosophila* H1 with anti-H1γ) (Table II). Anti-H1cs cross-reacts very strongly with H1γ, indicating that these two proteins share at least one common structural feature. In addition, the chromatographic behavior of H1cs on Bio-Rex 70 and Bio-Gel P-100 is very similar to that of other H1 subtypes. Thus, H1cs should be classified as an H1 histone. However, on the basis of its amino acid composition and electrophoretic properties, H1cs must differ substantially in primary structure from all H1 proteins described previously.

The two H1 subtypes expressed during the late stages of embryonic development, H1β and H1γ, are also unusual in that they show no cell cycle related phosphorylations (Cohen et al., 1979), which are exhibited by all other H1 proteins that have been examined (Gurley et al., 1975; Ajiro et al., 1981; Lennox et al., 1982). In addition, they are exceptionally tightly bound to chromatin (L. H. Cohen and K. A. Krzeminski, unpublished results). Their amino acid compositions clearly identify them as H1 histones, and the immunological data show that both of them are related to H1α, an H1 that is phosphorylated in a cell cycle dependent fashion (L. H. Cohen, R. Nardi, and G. M. McFadden, unpublished results).

The close agreement in amino acid composition between H1α and the H1 protein encoded in pSp2 is consistent with the stage specificity of expression of that gene (Levy et al., 1982). However, a few small differences were observed which may suggest the existence of heterogeneity or polymorphism of H1α.

The switches in H1 subtype expression observed in the sea urchin embryo correspond to times during development when there are also changes in chromatin structure (Arceci & Gross, 1980; Savic et al., 1981; Chambers et al., 1983). This is consistent with the involvement of H1 in various aspects of chromatin structure (Thoma et al., 1979; Allan et al., 1980; Stein & Kunzler, 1983). The striking differences between the sea urchin H1 subtypes, in their times of developmental expression, structural properties, and phosphorylation, suggest that these proteins are functionally different. In an effort to gain insight into this possibility, we are using immunological and biochemical techniques to study their distribution in the embryo and their arrangement in chromatin.

Acknowledgments

We thank Joselina Gatmaitan for assistance with amino acid analyses and Dr. Candido Alfageme for assistance with immunological procedures.

References

Ajiro, K., Borun, T. W., & Cohen, L. H. (1981) *Biochemistry 20*, 1445.

Allan, J., Hartman, P. G., Crane-Robinson, C., & Aviles, F. X. (1980) *Nature (London) 288*, 675.

Allan, J., Smith, B. J., Dunn, B., & Bustin, M. (1982) *J. Biol. Chem. 257*, 10533.

Arceci, R. J., & Gross, P. R. (1980) *Dev. Biol. 80*, 186.

Bustin, M., & Cole, R. D. (1968) *J. Biol. Chem. 243*, 4500.

Butler, P. J. G., & Thomas, J. O. (1980) *J. Mol. Biol. 140*, 505.

Chambers, S. A. M., Vaughn, J. P., & Shaw, B. R. (1983) *Biochemistry 22*, 5626.

Cohen, L. H., Newrock, K. M., & Zweidler, A. (1975) *Science (Washington, D.C.) 190*, 994.

Cohen, L. H., McFadden, G., & Nardi, R. (1979) *J. Cell Biol. 83*, 171a.

De Groot, P., Strickland, W. N., Brandt, W. F., & Von Holt, C. (1983) *Biochim. Biophys. Acta 747*, 276.

Finch, J. T., & Klug, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1897.

Gurley, L. R., Walters, R. A., & Tobey, R. A. (1975) *J. Biol. Chem. 250*, 3936.

Hawkes, R., Niday, E., & Gordon, J. (1982) *Anal. Biochem. 119*, 142.

Hewish, D. R., & Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun. 52*, 504.

Hill, R. J., Poccia, D. L., & Doty, P. (1971) *J. Mol. Biol. 61*, 445.

Hohmann, P., & Cole, R. D. (1971) *J. Mol. Biol. 58*, 533.

Kinkade, J. M., Jr. (1969) *J. Biol. Chem. 244*, 3375.

Kinkade, J. M., Jr., & Cole, R. D. (1966) *J. Biol. Chem. 241*, 5798.

Laemmli, U. (1970) *Nature (London) 227*, 680.

Lennox, R. W., & Cohen, L. H. (1983) *J. Biol. Chem. 258*, 262.

Lennox, R. W., Oshima, R. G., & Cohen, L. H. (1982) *J. Biol. Chem. 257*, 5183.

Levy, S., Sures, I., & Kedes, L. (1982) *J. Biol. Chem. 257*, 9438.

Newrock, K. M., Alfageme, C. R., Nardi, R. V., & Cohen, L. H. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 421.

Panyim, S., & Chalkley, R. (1969a) *Biochem. Biophys. Res. Commun. 37*, 1042.

Panyim, S., & Chalkley, R. (1969b) *Arch. Biochem. Biophys. 130*, 337.

Pehrson, J. R., & Cole, R. D. (1980) *Nature (London) 285*, 43.

Pehrson, J. R., & Cole, R. D. (1982) *Biochemistry 21*, 456.

Rall, S. C., & Cole, R. D. (1971) *J. Biol. Chem. 246*, 7175.

Ruderman, J. V., & Gross, P. R. (1974) *Dev. Biol. 36*, 286.

Salik, J., Herlands, L., Hoffmann, H. P., & Poccia, D. (1981) *J. Cell Biol. 90*, 385.

Savic, A., Richman, P., Williamson, P., & Poccia, D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3706.

Seyedin, S. M., & Kistler, W. S. (1980) *J. Biol. Chem. 255*, 5949.

Stein, A., & Künzler, P. (1983) *Nature (London) 302*, 548.

Stollar, D. D., & Ward, M. (1970) *J. Biol. Chem. 245*, 1261.

Thoma, F., & Koller, Th. (1977) *Cell (Cambridge, Mass.) 12*, 101.

Thoma, F., Koller, Th., & Klug, A. (1979) *J. Cell Biol. 83*, 403.