HISTONES AND THEIR MODIFICATIONS

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I. INTRODUCTION

In 1884, Albrecht Kossel[1,2] reported the isolation of an acid-extractable peptone-like component from goose erythrocyte nuclei. He named this component histone and suggested that it might be bound to nucleic acid. During the ensuing century, considerably more information has been obtained concerning roles of histones, the family of small basic proteins that bind to DNA in chromatin. Insight into the structural role of histones was obtained with the discovery of the repetitive structure of chromatin.[3-6] The unit which underlies this repetitive structure, now called the nucleosome, contains about 200 base pairs of DNA tightly complexed with an octamer of core histone molecules and more loosely associated with a linker histone.[7] The four-core histone species are known as H2A, H2B, H3, and H4. The linker histone species is known as H1 (including H1° and H5).

About the same time as the discovery of the nucleosome, electron microscopy provided visual evidence concerning the repetitive structure of chromatin.[8-10] Under certain conditions, chromatin could be seen as beads on a string. The beads, named nu (ν) bodies, had sizes and spacings consistent with those of the biochemically derived nucleosome. Nucleosome core particles as well as core histone octamers have been crystallized; thus this structural aspect is approaching at least a partial solution with the increasing resolution of the X-ray crystallographic studies.

The nucleosome model provided more insight into how chromatin is structured than how histones participate in the various functions of chromatin. Chromatin is involved directly in three major cellular functions: replication, transcription, and, in the germ cells, reproduction. Germ line functions include not only meiotic segregation but also the repackaging of the chromatin in spermatozoa. Studies on the mechanisms of most of these processes have been complicated by the continuous nature of DNA in which genes for completely unrelated functions as well as DNA which is never transcribed are all present in one very long continuous chemical entity. The availability of purified genes and the in vitro reconstruction of chromatin promises to provide much more precise knowledge of some of these processes in the near future.

The literature published prior to 1974 contained several nomenclatures for histone fractions: in that year at the Ciba Foundation Symposium on the structure and function of chromatin, a new nomenclature was proposed which has been universally accepted.[11]

The four core histones are as follows, with older nomenclatures in parentheses: H4 (F2a1, IV, GRK); H3 (F3, III, ARK); H2A (F2a2, IIb1, ALK); and H2B (F2b, IIb2, KSA). Lysine-rich histones commonly encountered in the literature are H1, (F1, I, KAP) and H5 (F2c, V, KAS).

However, over the last decade it has been found that each of the major histone fractions is composed of isoprotein species or variants. The variant designation is a number or letter which by convention follows the main fraction designation, separated by a period, i.e., H3.1 or H2A.X. In some cases different laboratories have used different isoprotein designations for what has turned out to be the same protein. Also, there is no guarantee that a variant designation of one laboratory corresponds to the same protein with the same designation from another laboratory since these may be assigned independently. In addition, there may not be universal agreement as to the relatedness of some histone variants. Thus the variant nomenclature is in a state of flux as variants are discovered in different species. The variants typically present in mammalian cells are discussed in Sections II and V.

In this review, we will first discuss the basic structural features of the histone proteins, sequences, modifications, and interactions with each other to form the nucleosome. We will then proceed to discuss the involvement of histones in the various functions of chromatin.

II. HISTONE EVOLUTION

Primary sequence analyses indicate all histones are rich in basic amino acids, lack tryptophan, and, as a general class of proteins, have highly conserved amino acid sequences.[12-14] The variations in primary structure that do occur fall into two categories. In the first category are those sequence variations resulting from the evolution of the eucaryotic species. These variations usually involve simple point mutations or deletions of one or several residues in closely related species and involve more extensive alterations in more distant species. The standard free energy of binding between histones derived from the same species or different species remains relatively constant, suggesting that the binding surfaces between histones have been highly conserved.[15-18] In the second category are those histone sequences that have varied within the same species; these can be further divided into three groups. One group of variants is associated with changes occurring over time such as during development; thus there are early and late histone variants in certain organisms.[19,20] A second group belongs to changes arising from terminal differentiation of specific tissues; thus there are sperm specific[21] and red blood cell specific variants.[22,23] Finally, there is a group of variants found in the same nucleus[24,25] or different nuclear compartments (*Tetrahymena*)[26] in a cell at the same time. The variations found in this second category may involve multiple insertions, i.e., complete changes in amino acids sequences with appearance of new domains in the protein molecule.[12-14]

New variants have generally been detected by one of two methods. The existence of some major variants was first demonstrated because of the presence of microheterogeneity at certain amino acid positions during sequencing. The existence of some minor variants was first demonstrated because of their differential interactions with the nonionic detergent, Triton® X-100, during polyacrylamide gel electrophoresis.[24,25] It is quite likely that there are additional variants as yet undiscovered.

A. Histone Proteins

1. H4

H4, an arginine-rich histone, generally has no variants. In *Tetrahymena*, two variants of H4 have been reported; the minor one (14% of the total H4) has an arginine insertion.[27] Representative sequences of H4 from the four eucaryotic kingdoms, animal, plant, fungi, and protista, are shown in Figure 1.[27-34] Of the five species of histones, H4 appears to be

HISTONE 4

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Bovine              SGRGKGGKGL GKGGAKRH-RK VLRDNIQGIT KPAIRRLARR GGVKRISGLI YEETRGVLKV FLENVIRDAV TYTEHAKRKT
Pea                 .......... ........-.. .......... .......... .......... .........I .......... ......R...
Yeast               .......... ........-.. I......... .......... .......... ...V.A...S ...S....S. ..........
Tetrahymena H4.1    A.-.......M ..V.....S.. SNKAS.E... .......... .......SF. .DDS.Q...S .....V.... ......R...
              .2    A.-.......M ..V.....S.. SNKAS.E... .......... .......SF. .DDS.Q...S .....V.... ......R...

Bovine              VTAMDVVY-AL KRQGRTLYGF GG  (102)
Pea                 .........-.. .......... ..  (102)
Yeast               ..SL....-.. .......... ..  (102)
Tetrahymena H4.1    .........-.. .......... ..  (102)
              .2    ........R.. .......... ..  (103)
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FIGURE 1. Sequences of H4. All sequences referenced to bovine. A space is skipped at every 10th residue of bovine sequence. Dots (. . .) mean the same as bovine. Dashes (- - -) mean a deletion due to alignment in that sequence. Number in parenthesis at the end of the sequence is the total number of amino acid residues in that sequence. Sequences or differences in sequences among variants were taken from References 28—30 (bovine), 31 (pea), 32 and 33 (yeast), and 27 and 34 (*Tetrahymena*).

the most conserved during evolution. H4 of pea plants differs from H4 of calf thymus by two conservative substitutions.[31] Sequences derived from other vertebrates, mouse[35] and *Xenopus*,[36] are identical to that of calf. Sequences from invertebrates, such as sea urchin[37-39] and starfish,[39] seem to have a specific substitution of a cysteine for a threonine at bovine sequence position 73. Because of this substitution, the H4 of invertebrates migrates more slowly on acid-urea-Triton® gels.[40] When fungi and protista are considered, the sequences of H4 do show more evolutionary divergence. Twenty amino acid residues differ between the calf and *Tetrahymena* sequences.[27] Most of those observed differences are internal and are not found in the amino- or carboxyl-terminal portions of the molecule. The carboxyl-terminal portion of H4 is involved in histone-histone interactions (see Section III). Thus, it is not surprising that these portions of the amino acid sequence have been more conserved. At present, no structural role has been demonstrated for the amino-terminal portion. The highly conservative nature of this region, however, does suggest that an important function does exist. Felsenfeld[41] has suggested that the amino-terminal portion of H4 may be involved in nucleosome-nucleosome interactions. It is interesting to note that the organism with the most divergent sequence also has a variant of H4. This higher degree of sequence divergence and the existence of H4 variants may be the result of the fact that *Tetrahymena* has two nuclei, micro and macro, with distinct biological functions.

Additional H4 sequences, whether complete or partial, are available for the following organisms: mouse,[35] human,[42,43] pig,[44] rat,[45] chicken,[46] *Xenopus*,[36] trout,[47] sea urchin,[37-39] fruit fly,[48] and wheat.[49,50]

2. *H3*

H3, another arginine-rich histone, is also highly conserved. The amino acid sequences determined directly by peptide sequencing or derived indirectly from the DNA sequences of cloned genes are shown in Figure 2. The three sequences of the bovine H3 variants[51-53] are fairly representative of the different H3 sequences found in the eucaryotic kingdom.[12-14] The sequences of the chicken H3 variants[54-57] are very similar to those of calf, with four substitutions. Five amino acid differences have been detected between pea[58] and cow. However, as one descends the evolutionary ladder, the number of differences increases. There are 15 amino acid substitutions between yeast H3[32,59] and bovine H3. Four of the substitutions require two base changes in the nucleotide sequence; the others are conservative. Of the 15 substitutions, 9 occur at the carboxyl-terminal third of the molecule. The replace-

HISTONE 3

Bovine	H3.1	ARTKQTARKS	TGGKAPRKQL	ATKAARKSAP	ATGGVKKPHR	YRPGTVALRE	IRRYQKSTEL	LIRKLPFQRL	VREIAQDFKT	
	.2	.....	.....	.....	.....	.....	.....	.....	.....	
	.3	.....	.....	.....	.....	.....	.....	.....	.....	
Mouse	.1	.....	.....	.....	.....	.....	.....	.....	.....	
	.2	.....	.....	.....	.....	.....	.....	.....	.....	
	.3	.....	.....	.....	.....	.....	.....	.....	.....	
(pseudo)	?	M.H.....	.C.....	.....?....	S.....S..	...D....L.	....-H....	..H.....	.Q.....	
Pea		.....	.....	.....	.....	F.....	..K.....	.....	.....	
Yeast		.....	.....	.S.....	S.....	.K.....	...F.....	.....	.....	
Tetrahymena	.1	.....	..A.....	.S.....	....I.....	F.....	..K.....D.	.....	..D..HE..A	
	.2	.....	..V.....	.....	VS.....K	F.....	..K...T.D.	.....	..D..MEM.N	

Bovine	H3.1	DLRFQSSAVM	ALQEACEAYL	VGLFEDTNLC	AIHAKRVTIM	PKDIQLARRI	RGERA	(135)		
	.2	.....	.....S....	.....	.....	.....	.....	(135)		
	.3	.....IG	.....S....	.....	.....	.....	.....	(135)		
Mouse	.1	.....	.....	.....	.....	.....	.....	(135)		
	.2	.....	.....S....	.....	.....	.....	.....	(135)		
	.3	.....IG	.....S....	.....	.....	.....	.....	(135)		
(pseudo)	?	......A.IG	.....S..NR	.....	.....C..VI	.....HS.	L....			
Pea		.....S	.....A....	.....	.....	.....	.....	(135)		
Yeast		.....IG	....SV....	.S.....	A.....	.....Q	K...K....L	....S	(135)	
Tetrahymena	.1	E.....L	.....A....	.....	....R.....	T..M.....	....F	(135)		
	.2	.I....Q.IL	.....A....	.....	....R.....	T..M.....	....F	(135)		

FIGURE 2. Sequences of H3. All sequences referenced to bovine. Microheterogeneity at position 127 of bovine and mouse sequences was reported by Franklin and Zweidler[24] (A/T, A shown only). A space is skipped at every 10th residue of bovine sequence. Dots (. . .) mean the same as bovine H3.1. Dashes (- - -) mean a deletion due to alignment in that sequence. Question marks (???) mean unknown or not determined. Number in parenthesis at the end of the sequence is the total number of amino acid residues in that sequence. Sequences or differences in sequences among variants were taken from References 24 and 51—53 (bovine), 24 and 62—65 (mouse), 58 (pea), 32, 33 and 59 (yeast), and 60 (*Tetrahymena*).

ment of proline and glutamine by two lysyl residues increases the positive charge of the yeast H3 molecule, resulting in the higher electrophoretic mobility of yeast H3 histone. The H3.1 and H3.2 variants of *Tetrahymena*[60] differ from the bovine H3.1 and H3.2 variants in a total of 17 and 21 positions, respectively. These numbers of differences are comparable to the 20 residue difference seen for H4 between *Tetrahymena* and cow. The 3 bovine variants differ from each other by 1 or 2 amino acids at positions 96, 89, and 90.[24,51-53] The substitution of serine for cysteine at position 96 has a large effect on detergent binding, resulting in a large difference in migration characteristics of H3.1 on acid-urea-Triton® X-100 gels.[24,56,61]

The fourth mouse sequence shown in Figure 2 is a sequence derived indirectly from the DNA sequence of a cloned gene.[62,63] This sequence is peculiar in that it is missing a glutamine at a highly conserved position and has many amino acid substitutions, especially replacement of histidines by arginines which would change the basicity of the histone molecule. Furthermore, the elimination of four arginyl residues in the carboxyl terminus effectively destroys one of the proposed DNA binding sites.[62] Yet, there is still a great deal of homology with the H3 sequence of known mouse variants.[14,64,65] The amino acids at positions 89 and 90 indicate that this mouse H3 gene should code for a H3.3 variant. It is presently unknown whether this gene is expressed because no such protein has ever been isolated. This mouse sequence may belong to the family of pseudogenes.[66] As more DNA sequences of cloned fragments become available it becomes increasingly important to demonstrate that the DNA sequence represents a functional protein. The yeast H3 amino acid sequence was derived indirectly from the DNA sequence of a cloned gene[32] and confirmed by others via peptide sequencing[59] to establish co-linearity of the map.

The mass content of each H3 variant may differ from cell to cell and tissue to tissue.[64,65] For example, H3.3 is the major variant in mouse liver while the H3.2 variant is the major variant in mouse L1210 cells. Humans and cows have much more H3.1 than H3.2. Zweidler[64] reported that the mass fraction of H3.3 in H3 increases from 15% in livers of newborn mice

to 55% in livers of adult mice, showing that variant patterns may change with age. Wu et al.[67] have reported that H3.3 is the major H3 variant in quiescent human lymphocytes. However, by 3 days after activation with phytohemagglutinin, H3.1 and H3.2 have become the major variants (see Section V).

In *Tetrahymena*, a minor H3 variant, hv2, has been isolated from the macronuclei; it is missing from the micronuclei.[68] Hayashi et al.[60] suggest that the minor H3.2 variant listed in Figure 2 is the hv2 protein isolated by Allis et al.[68] Other H3 sequences, whether complete or partial, are available for the following organisms: trout,[69,70] shark,[71] small mouth buffalo fish,[72] sea urchin,[37,73,74] fruit fly,[48] *Xenopus*,[36] chicken,[54-57] human,[75,76] and wheat.[77]

3. H2B

H2B sequences have diverged more during evolution than those of histone H3 and H4. The H2B molecule can be divided into two readily identifiable domains, the amino terminus where lysine and arginine are the predominant amino acids and a large hydrophobic domain containing the carboxyl terminus. The sequence of the amino-terminal third is highly divergent while that of the carboxyl two thirds is highly conserved (Figure 3).[12-14,63,78-80] Differences in the amino-terminal domain may involve insertions and deletions. Especially noticeable in sea urchin sperm H2B,[81,82] but also readily discernable in other species,[79] are several to many small repeating pentapeptide structures beginning with proline and containing one to three charged amino acids that have been inserted into the amino termini.[13,82]

A complete H2B sequence from the plant kingdom is not currently available. The pea H2B has been cleaved by BrCN and trypsin and the fragments of the carboxyl-terminal region align with the bovine sequence.[83] Similarly, the carboxyl-terminal region of wheat has been sequenced and is presented in Figure 3.[13] The changes in the carboxyl-terminal domain are mostly conservative in nature.

The number of H2B variants differs from species to species. According to Zweidler,[84] two major variants (H2B.1 and H2B.2) and one minor variant (H2B.3) can be detected on polyacrylamide gels in acid extracts of mouse tissues. However, in other mammals and in avians, there seems to be no equivalent H2B.2 to the mouse H2B.2. However, they do have the minor variant also designated H2B.2 that is equivalent to mouse H2B.3. Thus, it is important to note the animal species when comparing sequences (sequence of H2B.2 mouse $\neq$ sequence of H2B.2 cow).

Additional H2B sequences, whether complete or partial, are available from the following organisms: human,[85] chicken,[86,87] *Xenopus*,[88] trout,[89] fruit fly,[90] sea urchin,[37,73,81] starfish,[91] mollusk,[92] and crocodile.[88]

4. H2A

Histone 2A has diverged the most during evolution and has the most variant forms of the core histones (Figure 4).[12-14,93-105] In mammalian cells, West and Bonner[25,99] have described four variants, named H2A.1, H2A.2, H2A.X, and H2A.Z. The four proteins were shown to be H2A variants by several criteria. All four proteins contained the conserved H2A sequence AGLOFVGRV (Figure 4). The K/R ratios of the four proteins were similar to each other, but very different from those of other core histones. All four proteins could be modified by the covalent attachment of ubiquitin. Zweidler[84] has given alternative nomenclature to H2A.X and H2A.Z. He named them, respectively, H2A.4 and M1. Furthermore, he considers M1 to be a minor histone-like protein in a distinct class of molecules not in the H2A family.

The sequences of the major variants, H2A.1 and H2A.2 of vertebrates, are nearly identical to each other with the exception of a few conservative substitutions.[12-14] The substitution of methionine for leucine at position 51 of cow seems to be the variation in sequence that differentiates H2A.2 from H2A.1 in all vertebrates.[14] Substitution at other positions depends

HISTONE 2B

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Bovine        H2B.1  ----- PEPAKSAPA---P KKGS-----KKAVTK AQKKDGKKRK RSRKESYSVY VYKVLKQVHP DTGISSKAMG IMNSFVNDIF
                 .2  ----- .........---. ....-----..... .......... .......... .......... .......... ..........
Mouse            .1  ----- .........---. ....-----..... .......... .......... .......... .......... ..........
                 .2  ----- .........---. ....-----..... .......... .......... .......... .......... ..........
                 .3  ----- .........---. ....-----..... .......... .......... .......... .......... ..........
Yeast            .1  SAKAE KK..SK...EKK. AAKKTSTST------ ----.....S KA...T..S. I......T.. .....Q.S.S .L........
                 .2  SSAAE KK..SK...EKK. AAKKTSTSV------ ----.....S KV...T..S. I......T.. .....Q.S.S .L........
Tetrahymena          ----- .KK.PA.-.---E ..V-------...P.- TE..N-..-. ..--.TFAI. IF..... .V...K.A.N .....I..S.
Wheat                ????? ???????????? ???????????????? ?????????? ?????T.K1. IF..... .1.......S .....1....

Bovine        H2B.1  ERIAGEASRL AHYNKRSTIT SREIQTAVRL LLPGELAKHA VSEGTKAVTK YTSSK--  (125)
                 .2  .....Q.... .......... .......... .......... .......... .....--  (125)
Mouse            .1  .......... .......... .......... .......... .......... .....--  (125)
                 .2  ....S..... .......... .......... .......... .......... .....--  (125)
                 .3  .....Q.... .......... .......... .......... .......... .....--  (125)
Yeast            .1  ....T...K. .A...K...S A......... I......... .....R.... .S..TQA  (130)
                 .2  ....T...K. .A...K...S A......... I......... .....R.... .S..TQA  (130)
Tetrahymena          ....L.S.K. VRF...R.LS ...V....K. .......R.. I......... FS..TN-  (119)
Wheat                .KL...SAK. .R...KP... ......S... V......... .......... F..A.--
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FIGURE 3. Sequences of H2B. All sequences referenced to bovine. A space is skipped at every 10th residue of bovine sequence. Dots (. . .) mean the same as bovine H2B.1 Dashes (- - -) mean a deletion due to alignment in that sequence. Question marks (???) mean unknown or not determined. Number in parenthesis at the end of the sequence is the total number of amino acid residues in that sequence. Sequences or differences in sequences among variants were taken from References 14, 24 and 78 (bovine), 14, 24 and 63 (mouse), 33 and 79 (yeast), 80 (*Tetrahymena*), and 13 (wheat).

HISTONE 2A

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Bovine        H2A.1  -------SGRGKQGGKA --RAK-A--K--TRSSR AGLQFPVGRV HRLLRKGNYA E-RVGAGAPVY LAAVLEYLTA EILELAGNAA
                 .2  ---------------- --...-.--.--S.... .......... .......... .-......... M.......... ..........
                 .Z  -----AGGKA..DS... --KT.-.----VS..Q. ........?? ?????????? ?-????????? ?????????? ??????????
Mouse            .1  ---------------- --...-.--.--..... .......... .......... .-......... .......... ..........
                 .2  ---------------- --...-.--.--S.... .......... .......... N-......... M.......... ..........
                 .X  --------...??????? --???-?--?--??... .......... .....????? ?-?........ .......... ..........
                 .Z  -----???????????? --???-?--?--????? .........? ?????????? ?-????????? ?????????? ??????????
Chicken          .1  ---------------- --...-.--.--S.... .......... .......... .-......... .......... ..........
                 .2  ----------.....V --...-.--.--S.... .......... .......... .-......... M.......... ..........
                 .F  -----AGGKA..DS... --K...-.----VS..Q. .........I ..H.KTRTTS HG....T.A.. S..I..... .V.......S
Yeast            .1  -------..-..-.... GSA..-.S--Q-S..AK ...T..... .....R.... Q-.I.S..... .T......A. ..........
                 .2  -------..-..-.... GSA..-.S--Q-S..AK ...T..... .....R.... Q-.I.S..... .T......A. ..........
Tetrahymena      .1  -------.TT..-.... --KG.T.SS.QVS..A. .........I S.F.KH.R.S .-.I.T..... ........A. .V........
                 .2  -------.TT..-.... --KG.T.SS.QVS..A. .........I S.F.KN.R.S .-.I.T..... ........A. .V........
Wheat            .1  ---------A.RK..-D --.K.-.----V...VK .........I G.Y.K..R.. Q-...S..... .......??? ??????????
                 .2  MDGSKLKKVAA.KF.-- GP.K.--S---V.K.IK .........I ?????????? ?????????? ?????????? ??????????
                 .3  MDASKAKKVA..KF.-- GP.K.--S---V...IK .........I ?????????? ?????????? ?????????? ??????????

Bovine        H2A.1  RDNKKTRIIP RHLQLAIRND EELNKLLGKV TIAQGGVLPN IQAVLLP---KKT ESHHKAKGK-  (129)
                 .2  .......... .......... .......... .......... .......---... .........-  (129)
                 .Z  ?????????? ?????????? ?????????? ?????????? ???????---??? ??????????-
Mouse            .1  .......... .......... ........R. .......... .......---... .........-  (129)
                 .2  .......... .......... .......... .......... .......---... .........-  (129)
                 .X  .....??... .......... ........G. .......... .......---.?? ??????????-
                 .Z  ?????????? ?.......?? ?????????? ?????????? ???????---??? ??????????-
Chicken          .1  .......... .......... .......... .......... .......---... D..-...A.-  (128)
                 .2  .......... .......... .......... .......... .......---... ...-...S.-  (128)
                 .F  K.L.VK..T. ........G. ...DS.I-.A ...G...I.H .HKS.IG---..G QQK-T.----  (127)
Yeast            .1  .......... .......... D.......N. .......... .HQN...---..S AKAT..SQEL  (131)
                 .2  .......... .......... D.......N. .......... .HQN...---..S AKTA..SQEL  (131)
Tetrahymena      .1  K.......V. ..IL..... ......MANT ...D..... .NPM...SKS... ..RGQ.SQDI  (137)
                 .2  K.......V. ..IL..... ......MANT ...D..... .NPM...SKT... SEAEH-----  (132)
Wheat            .1  ?????????? ?????????? ?????????? ?????????? ???????????????? ??????????
                 .2  ?????????? ?????????? ?????????? ?????????? ???????????????? ??????????
                 .3  ?????????? ?????????? ?????????? ?????????? ???????????????? ??????????
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FIGURE 4. Sequences of H2A. All sequences referenced to bovine. A space is skipped at every 10th residue of bovine sequence. Dots (. . .) mean the same as bovine H2A.1. Dashes (- - -) mean a deletion due to alignment in that sequence. Question marks (???) mean unknown or not determined. Number in parenthesis at the end of the sequence is the total number of amino acid residues in that sequence. Sequences or differences in sequences among variants were taken from References 14, 24, and 93—97 (bovine), 14, 24, 98, and 99 (mouse), 14, 56, and 100—102 (chicken), 33, 79 and 103 (yeast), 104 (*Tetrahymena*), and 105 (wheat).

on the species and may represent allowable sequence diversity during evolution of the species. The H2As of the other eucaryotic kingdoms show much more variation in their sequences relative to the bovine sequences. Numerous changes are evident at both the amino and carboxyl termini of the H2A molecules, including additional amino acids at the amino-terminus insertions, deletions, and nonconservative substitutions. The numerous changes between positions 8 and 16 (Figure 4) seem to be characteristic of invertebrates.[12-14] Furthermore, in the case of insertions, only a few selected amino acids are allowed at these positions. In contrast, the lysine at position 13 is invariant and is found in all the H2As, thus implying strong selective pressures to maintain the sequence at this position.

The minor histone variants H2A.Z and H2A.X represent 5 to 20% of the histone H2As in vertebrates.[25] From SDS-polyacrylamide gel analysis of mouse H2As, the apparent molecular weight of H2A.Z is 600 daltons smaller and that of H2A.X is 1000 daltons larger than that of H2A.1. H2A.Z of cow[97] and H2A.F of chicken[102] appear to be the same or closely related proteins. They have 29 of the first 30 amino acids in common, and a protein with the same migration characteristics as H2A.Z is known to exist in avian cells.[56] Of course, a caveat of this view is that the H2A.F amino acid sequence is derived indirectly from the DNA sequence of a cloned chicken gene. Additional protein sequencing data are needed to make a definitive judgment. It is interesting to note that both H2A.Z and H2A.F have the valyl insertion (between #15 and 16 of bovine H2A.1 sequence) that is common to representatives of the other three kingdoms, suggesting H2A.Z may be a more primitive form of H2A. This view is further supported by comparing some of the biochemical characteristics of H2A.Z from different species. H2A.Z from sea urchin co-migrates with mouse H2A.Z in 2D gels, whereas all other H2As migrate differently.[40] Peptide mapping demonstrated that the H2A.Zs from mouse and sea urchin have seven arginine and eight lysine-containing peptides in common, whereas the H2A.2 of mouse and the H2A.α of sea urchin differ at many positions.[40] H2A.Z also differs considerably from other H2As found in the chromatin of the same nucleus.[99] For example, H2A.Z does not have sulfur-containing amino acids in any of its peptides and has only a 60% sequence homology with H2A.1 in the amino-terminal domain. H2A.Z has a two-residue extension and 10 amino acid substitutions all within the first 21 amino acid residues. These substitutions result in a change in net charge of the amino-terminal domain in H2A.Z to +6 as opposed to +8 for the corresponding region of H2A.1. Furthermore, one of the substitutions removed the serine at the amino terminus of H2A.1, the site of phosphorylation of other H2As. A total of only two arginine and no lysine-containing peptides were found to be common between H2A.1 and H2A.Z of mouse. Thus, the H2A.Z variant is clearly much more conserved evolutionarily than the other H2As; yet at the same time, it is quite different from the other H2As in the same organism. In fact, the conservative nature of the H2A.Z sequence across species seems to be more like that of the H4s and H3s.

Other interesting features relevant to H2A.Z come from studies on sea urchin embryos,[40] *Tetrahymena*,[106] and *Drosophila*.[107-109] H2A.Z is the only H2A variant in sea urchins that does not seem to be developmentally stage specific; it is synthesized throughout early development.[40] Readers are referred to Maxson et al.[20] for recent reviews on the complex subject of patterns of histone changes during the course of early and late development. In *Tetrahymena*, a minor H2A variant named hv1 has been isolated from the macronuclei; it is missing from the micronuclei.[106] Furthermore, by immunological staining hv1 seems to be enriched in nucleolar chromatin.[110] Recently, Blumenfeld and co-workers[108,109] established that a minor *Drosophila* histone-like protein, D2,[107] was an H2A variant. They further showed by indirect immunofluorescence that antibodies against D2 bound to the interband regions of polytene chromosomes,[109] suggesting that this protein may be involved in transcription. It has been suggested that the *Tetrahymena*[106,110] and *Drosophila*[108,109] proteins may be functionally similar to mammalian H2A.Z.

Recently, Bhatnagar et al.[111] reported that H2A.X was also enriched in nucleolar chromatin of several mouse tissues.

Other H2A sequences, whether complete or partial, are available for the following organisms: sea urchin,[37,59,74,112,113] cuttle fish,[114] trout,[70,115] starfish,[116] fruit fly,[48] *Sipunculus nudus* (a marine worm),[117] rat,[118] and human.[119]

5. H1, H1°, and H5

The outer or linker histones found in chromatin are members of a family consisting of H1, H1°, H5, and their variants.[120] H1 has been found in all cells with the exception of yeasts. H1° is found in high amounts in some quiescent cells[121] and H5 is only found in avian red cells.[22,23] The number of H1 variants and the amount of each variant can differ from tissue to tissue, and for a given tissue can differ from one species to another.[120,122,123] Rall and Cole[124] have reported different forms of H1 for rabbit and calf. Recently, Lennox and Cohen[123] reported the electrophoretic separation of mouse H1 into five different forms. Smith and Johns[121] had previously reported that H1° can be separated into two variant forms. In the case of *Tetrahymena*, the macronucleus has a typical H1 like other eucaryotes.[125] The micronucleus, however, does not contain any detectable macronuclear type H1. Instead, three peptides (α, β,γ) replace the H1 on the linker region of DNA.[125]

The nomenclature for H1 and H1° variants has not been codified. The relationship between one system of naming variants to another system has not been clearly defined. Thus, H1.1 in one system may not be the same as H1a in another system. We have chosen to use the designations given by the original authors in tabulating the sequences of H1 (Figure 5). The chicken H1 (DNA) sequence was indirectly derived from the DNA sequence of a cloned gene.[46] The rabbit H1.3, the pig H1t, and the chicken H5 are the only sequences in Figure 5 that have been totally sequenced using classical peptide sequencing techniques.[124,126,134] Rall and Cole[124] had shown some possible alignments of tryptic peptides derived from RTL-4 and CTL-1 that were based only on partial sequencing of nonoverlapping peptides. These alignments have found their way into the protein sequence data bank and some review articles as established authentic sequences. Cole[129] has noticed these errors in interpretation and has kindly provided the correct sequences for cow H1.1, H1.2, H1.3a, and H1.3b; and rabbit H1.0a, H1.2, H1.3, and H1.4. These sequences have all been properly sequenced and overlapped.[124,127,128,133,135]

As a class of histones, H1 is the most evolutionarily variable. However, it does have a centrally located globular region of highly conserved sequence beginning with the 41st residue of bovine H2A.1 sequence. Both the amino and carboxyl termini are highly variable and are very basic. The detergent-binding properties of H1 are very different from the core histones. H1 interacts weakly, if at all, with nonionic detergents and thus remains on the diagonal in the 2D gel system of Bonner et al.[61] The H1 variants may, however, be separated from each other based on their size. Within the same cell, one variant may differ from another by about 1000 daltons. This difference resides in the carboxyl-terminal half of the primary structure.

H1° is more like H5 in its sequence.[131,132] The one CNBr peptide that has been sequenced from H1° of cow and pig has a sequence very similar to H5 in the conserved hydrophobic domain. It has been suggested by Smith et al.[131] that this conserved hydrophobic domain may be responsible for repressing DNA synthesis since both H1° and H5 are found in cells that do not replicate their DNA.

Additional H1 and H5 sequences, either complete or partial, from other organisms are available for the following: for H1, trout,[136] fruit fly,[48] sea urchin;[137,138] for H5, goose and pigeon.[139,140] Recently, Carozzi et al.[141] reported a partial DNA sequence of a gene coding for human H1.

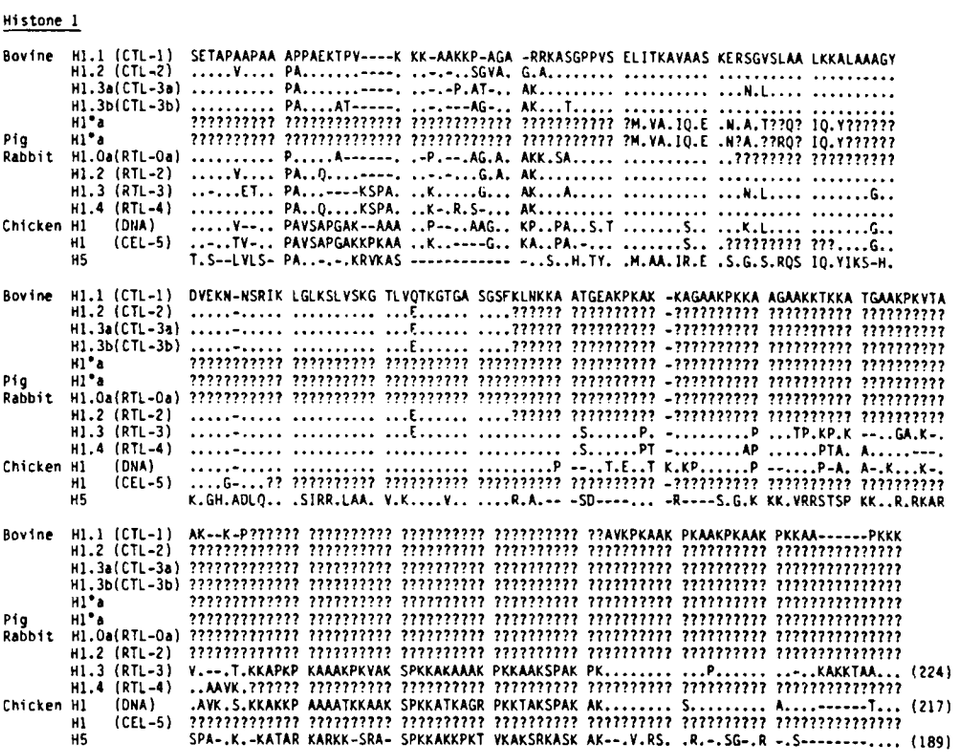

FIGURE 5. Sequences of H1, H1°, and H5. All sequences referenced to bovine H1.1 (CTL-1). A space is skipped at every 10th residue of bovine sequence. Microheterogeneity was detected in the following positions: #21 of bovine H1.3b (K/P, K shown only); #6, 18, and 21 of rabbit H1.0a (A/V, A shown only), (K/P, K shown only), (A/P, A shown only); #23 and #26 of rabbit H1.2 (A/V, A shown only), (P/T, P shown only); #78 of rabbit H1.4 (E/Q, E shown only), #99 of rabbit H1.4 (E/Q, Q shown only), #14 of chicken H5 (Q/R, R shown only). Dots (. . .) mean the same as bovine H1.1. Dashes (- - -) mean a deletion due to alignment in that sequence. Question marks (???) mean unknown or not determined. Number in parenthesis at the end of the sequence is the total number of amino acid residues in that sequence. Sequences or differences in sequences among variants were taken from References 128—130 and 133 (bovine), 132 and 134 (pig), 121, 124, 132, and 135 (rabbit), and 13, 46, 126, 131, and 139 (chicken).

B. Functional Significance of Histone Variants

The discovery of histone variants that are stably incorporated into nucleosomes makes possible many apparently different types of nucleosomes with different core as well as linker histone compositions without even considering the DNA sequence. Furthermore, the timing of the synthesis of the basal variants (see Section V) relative to the S-phase variants may lead to nonrandom localization of specific types of core particles in chromatin, thereby producing nucleosome heterogeneity with possible functional consequences. Two opposing positions can be taken on the question of functional significance of histone variants. One extreme position is that variants are merely the allowable diversity in sequence without affecting the same set of functions. The opposite position is that variants have significantly different functions although these function have not been discovered. Experimental evidence suggests that both positions may be correct in different situations.

The best evidence for nonfunctionality comes from the work by Grunstein and co-workers[79,142-144] on yeast. As previously described, yeast chromosomes contain histone H2A and H2B with variants for each of these proteins.[143] Grunstein and co-workers[79,142] constructed frame shift mutants at each of the H2B and H2A loci and studied whether each of the subtypes needed to be used differentially or whether one variant could replace the other.

They concluded that one functional H2A and one functional H2B protein must be present for nucleosome formation or else the mutation is a lethal event.[79,142] The respective H2As and H2Bs however can be interchanged or substituted for each other and, within the limits of the assay, there seems to be no exclusive interaction between any particular H2A and H2B subtype.[79,142] Nature also has performed a similar experiment with the H2B variants. The mouse has two major H2B variants, while most other mammals have only one.

Further evidence that suggests that differences in the primary structure of histones do not have functional significance comes from the work reported by Wallis et al.[144] They constructed a number of mutations in the amino terminus of the yeast H2B.2 gene. These mutations show that the proline pentapeptide repeat (Figure 3, proline at yeast positions 8, 13, and 18) is not an essential feature of H2B protein. An H2B protein can function with 4, 3, 2, 1, or none of the pentapeptide units.[79,144] Furthermore, deletions in the regions containing amino acid residues 2 to 22 and 10 to 51 of yeast sequence are not lethal. Larger deletions involving residues 17 to 70 are lethal. During chromatin assembly, the mutant proteins however are not selected against. These results may be rationalized in that the amino terminus of H2B resides on the outside of the octamer and has little role in the formation and maintenance of the nucleosome structure. However, the results do present a paradox in that this amino-terminal domain has not been deleted and in fact its sequence is well conserved in variants even though it does not seem to serve any obvious purpose in chromatin.

Recent experiments by Moreland and Hereford[145] may have shed some light on this paradox. They have shown that the amino terminus of H2B contains a signaling sequence for the intracellular transport of histone into nuclei. However, deletions in that region of the sequence were not lethal. They reasoned that the H2B might possibly be transported into the nucleus as a dimer with H2A, the H2A supplying the required sequence. A similar sequence has been identified in the SV40 large T antigen.[146]

The evidence in favor of specific functions is a collection of suggestive results from many sources. First, the yeast experiments cannot diminish the fact that there are whole sets of developmentally regulated histone subtypes in organisms such as the sea urchin. Simpson and co-workers[147,148] have compared the static and dynamic structural properties of chromatin isolated from adult, embryonic, and specialized tissues of sea urchins. They have shown that different variant compositions definitely have structural and possibly functional consequences. Chromatin isolated from sperm[148] and from early or late embryos[147] was shown to have different unfolding parameters at low ionic strength as well as different nuclease susceptibilities. These results suggest that perhaps different histone variant compositions may be needed for chromatin remodeling at different stages of development. Second, the existence of different variants in the different nuclear compartments of *Tetrahymena*[125] supports the observation that these variants may be responsible for the distinct separation of biological functions of the macro- and micronuclei, respectively, into a transcriptionally active compartment and a transcriptionally inactive compartment providing genetic continuity. Third, the existence of H5 in mature avian red cells, but not in other somatic cells,[22] and the existence of families of H1 proteins in the macro- and micronucleus of *Tetrahymena* are consistent with a repressive role on transcription by H1 variants.[125] Fourth, the highly conservative nature of H2A.Z and the small but rather constant amount of H2A.Z in all cell types and species in which it has been sought (5 to 10% of the total H2As) suggest a differentiative role for H2A.Z.[25]

Recently, Blumenfeld and co-workers[108,109] have shown that D2,[107] a minor H2A variant in *Drosophila* with properties similar to H2A.Z of higher eucaryotes, was present in the interband but not the band region of polytene chromosomes. However, D2 seemed to be absent from puffed regions, suggesting an involvement with the transcriptional processes occurring in puffs.

For other H2A variants, Blankstein and Levy[149] showed that the H2A.2/H2A.1 ratio of different Friend erythroleukemic cell lines correlated with the dimethylsulfoxide inducibility of those lines. Finally, cells in different physiological states have different histone-variant synthesis patterns (see Section V). Thus, S-phase cells synthesize all histone variants and G0 and G1 cells synthesize a subset of these variants. This synthesis of different histone variants suggests a continuous process of chromatin remodeling whether histones are required for replication or for turnover and replacement. The availability of specific histone variants at particular times or situations may be involved in the entrance into or exit from particular proliferative states.

As will be discussed in Section V, histone variants may be useful experimental tools irrespective of whether or not they exhibit functional differentiation.

C. Histone-Like Proteins in Prokaryotes

Although histones are eucaryotic proteins, proteins with histone-like properties have been found in prokaryotes. In 1976, Griffith demonstrated the possibility of DNA packaging in prokaryotes when *Escherichia coli* cells were lysed directly onto electron microscope grids in the presence of 0.15 *M* NaCl.[150] After dehydration and processing, electron microscopic observation indicated that the fibers of *E. coli* DNA had a 130 Å-repeating beaded structure. The possibility existed, however, that these structures were an artifact of the dehydration of DNA in the presence of salt since naked DNA when dehydrated onto a grid could be shown to produce similar structures.[151]

The existence of histone-like DNA-binding proteins was first brought to light by the studies of Rouviere-Yaniv and Gros.[152,153] They identified a protein which they designated "HU" (which others have also called HD, NS, protein 2, HLP2, and DNA-binding protein II, reviewed in Reference 154) which binds to both single- and double-stranded DNA and stabilizes the double-stranded DNA against thermal denaturation. It was also found that the incubation of this protein with relaxed circular SV40 DNA (in the presence of pure nicking-closing enzyme) caused the introduction of up to 18 negative superhelical turns in the DNA molecules as measured by agarose gel electrophoresis.[155] An HU/DNA mass ratio of one produced the greatest degree of supercoiling. This protein was found to be highly conserved in prokaryotes and immunologically cross-reacting proteins were found in a species of blue-green algae.[156] Amino acid sequencing later confirmed the significant conservation of sequence in these proteins.[157-160] Recently, Briat et al.[161] demonstrated that antisera prepared against the bacterial HU (DNA-binding protein II) cross-reacts with a DNA-binding protein which co-sediments with the nucleoid of spinach chloroplasts. Antibodies elicited against the HU protein of photosynthetic cyanobacteria were found to be more reactive than those raised against the *E. coli* HU protein. Like the HU protein of *E. coli*, the HU protein of chloroplasts was found to be a heterotypic dimer.[162]

Varshavsky et al.[163] used conditions similar to those used for the isolation of eucaryotic chromatin to isolate an *E. coli* chromosome which contained, in addition to RNA polymerase, two histone-similar proteins with apparent molecular weights of 17,000 and 9,000 daltons. The latter protein is equivalent to protein HU. They found that these two proteins occurred in approximately equimolar amounts. Digestion of the chromosomes yielded a fraction of subparticles containing approximately 120 base pairs and both of the aforementioned proteins. Lathe et al.[164] conducted genetic analysis of fir A mutations of *E. coli*, mutations which cause transcriptional thermosensitivity and abnormal RNA polymerase sensitivity to rifampicin. They concluded that the product of the fir A gene was most probably the 17,000 dalton DNA-binding protein since mutation at the fir A chromosomal region appeared to eliminate this protein. They found that this protein, which they termed HLPI, resembled histones in its charge, acid solubility, heat stability, ability to bind to DNA, and its lack of binding to hydroxylapatite. They suggested that the characteristic defects of the fir A mutants indicated an ability of this 17,000 dalton protein to interact with and stabilize RNA polymerase.

Two other prokaryotic histone-similar proteins have been isolated and termed H1 and H.[165,166] The 15,000 dalton H1 protein is composed of three forms differing in their isoelectric point. One form, H1a, has been shown to bind to DNA and cause its effective compaction with little concomitant positive supercoiling of the DNA.[167] The 28,000 dalton H protein is notable not only for its DNA-binding properties, but also for the similarity of its amino acid composition with that of histone H2A and for the ability of anti-H2A antibodies to neutralize its function.[166]

The stabilization of DNA of the free-living thermophilic archaebacterium *Thermoplasma acidophilum*, which grows in extreme conditions of heat and acidity, was studied by Searcy and co-workers.[168-171] Its DNA is stabilized by a small, tightly bound, acid-soluble protein (termed HTa) which resembles the eucaryotic histones in both size (two forms of 89 and 90 amino acids each), and amino acid composition. Unlike HU-DNA complexes which are stable only in low ionic strength conditions, this protein was found to be tightly bound to DNA in salt concentrations up to about 0.6 *M*. An additional contrast is the ability of this protein when bound to DNA to inhibit transcription by *E. coli* RNA polymerase. Cross-linking experiments indicated that monomers of HTa probably associate in vivo to form tetrameric complexes and these complexes were the unit of association with the DNA. Electron microscopic analysis showed that an individual globular unit of protein (tetramer of HTa), when associated with the DNA, reduced its contour length by 40 base pairs.

Peptide sequencing has shed more light on the interesting possibility of an evolutionary relationship of this protein to histone. HTa has 22% basic amino acid residues, while the four core histones together average 23 to 26% in basic residues, and the lysine to arginine ratio is 2.3, which compares closely to the value of 2.5 for calf histone H2B.[172,173] Notably, this protein has no tryptophan, cysteine, or histidine, and its 89-residue form has no methionine.

The complete amino acid sequence of HTa shows that this protein resembles the histones in having clusters of basic amino acids and a distinctive acidic region, but it is dissimilar in that the amino-terminal region is not highly basic.[174] Although a direct amino acid sequence comparison of the prokaryotic HU proteins with histones has failed to demonstrate any significant homology between the two, DeLange et al.[174] have identified statistically significant homologies of the HTa peptide sequence to regions in both the HU and the histone proteins.

Perhaps the relationship between histone-like proteins in prokaryotes and histones is a case of convergent evolution rather than conservation. By understanding the functions of the prokaryotic proteins, one may gain some useful insight into some aspects of histone function.

III. HISTONE INTERACTIONS

Over the past decade, a great wealth of experimental data has been accumulated on the organization of histones and DNA in chromatin. A large percentage of the relevant studies have already been reviewed,[7] so this discussion will be limited to a review of the more historically significant observations and to an update on the more recent findings.

Woodcock[8] and Olins[9,10] were the first to discover that chromatin can be visualized in the electron microscope as a string of bead-like particles. Brief digestion of eucaryotic chromatin with micrococcal nuclease yields particles called nucleosomes which contain about 200 base pairs of DNA associated with two each of the four core histones H2A, H2B, H3, and H4 and one molecule of histone H1 (reviewed in Reference 7). Further nuclease digestion brings about the production of the core nucleosome particle which consists of 146 base pairs of DNA wrapped around the core histones. An intermediate, transiently formed particle contains 166 base pairs of DNA with 2 each of the core histones and 1 molecule of histone H1. This particle, called the chromatosome, is thought to have histone H1 associated with

ten base pairs at each end of the DNA.[175] Within these nucleosomal structures, DNA sites are found to be exposed to the action of various nucleases at ten nucleotide intervals, with some site-specific degrees of accessibility along the length of the DNA.[4,7,176-182]

A. Biochemical Studies on Nucleosome Organization

The core histones which form the protein moiety of the nucleosome have been found to interact with each other in very specific ways. Kelley[183] discovered that histones H2A and H2B could interact with each other in a 1:1 stoichiometry as a dimeric complex, and Roark and co-workers[184,185] identified a tetrameric complex which contained two each of histones H3 and H4. D'Anna and Isenberg[16,186-188] were the first to systematically study the association constants of specific histone pairs. They were able to classify three levels in the hierarchy of strength of interaction between pairs of histones in solution. H2A-H2B, H2B-H4, and H3-H4 formed the strongest pairs; H2A-H4 and H2B-H3 formed the weakest pairs; and H2A-H3 formed a pair with an intermediate strength of association. Their in vitro physicochemical analysis of the interactions between calf thymus histones was repeated and confirmed for the histones of the pea plant,[189] yeast,[18] and *Tetrahymena*,[190] as well as for heterospecific mixtures of histones.[18,189,190]

Although these interactions were studied on isolated and purified pairs of proteins, a great body of literature on histone pairs linked together during chemical cross-linking of the proteins in chromatin and nuclei supports the presence of these histone pairs in vivo (reviewed in References 7 and 12). A major product of chemical cross–linking of chromatin with dimethylsuberimidate was identified as a complex which contained two each of the four core histones.[191-194] Analysis of the products of the chemical cross-linking released from the chromatin in 2 *M* NaCl at pH 9 led the authors to propose that the histones were released from the chromatin as an octameric complex. Through the combined use of cleavable cross-linking reagents and two-dimensional gel analysis they were able to more conclusively state that the octameric complex contained two each of the core histones. Thomas and Butler,[195] using sedimentation equilibrium, were able to determine that the molecular weight of the histone complex released from chromatin in 2 *M* NaCl at pH 9 was 107,500 daltons, a value consistent with an octameric structure of the form $(H2A)_2$ $(H2B)_2$ $(H3)_2$ $(H4)_2$.

Although the idea of participation of an octamer of the four core histones within the nucleosome was readily accepted, the actual state of interaction of these histones when extracted from chromatin in 2 *M* NaCl differed in the hands of different investigators.[195-201] Eickbush and Moudrianakis[201] investigated histone association in considerable detail using the techniques of gel filtration, sedimentation velocity, and sedimentation equilibrium. It was demonstrated that the histone complex released from chromatin in 2 *M* NaCl at pH 7.5 contained 2 each of the 4 core histones, and that this octameric complex was formed by the association of 2 H2A-H2B dimers and 1 $(H3\text{-}H4)_2$ tetramer. The equilibrium of the octamer with its tetramer and dimer subunits was affected by the ionic strength, pH, temperature, and presence of urea. The octamer can be formed by the association of $(H3\text{-}H4)_2$ tetramers and H2A-H2B dimers which contains the full spectrum of histone variant forms as well as their post-translationally modified and partially proteolyzed forms.[202]

The role of specific domains of the polypeptides in histone-histone interactions was delineated by Bradbury and co-workers,[203,204] who utilized NMR to study the ability of specific fragments of the core histones to interact. They determined that residues 31 to 95 of H2A and 37 to 114 of H2B are involved in the formation of the H2A-H2B dimer, while residues 42 to 120 of H3 and 38 to 102 of H4 are involved in the tertiary structure of the $(H3\text{-}H4)_2$ tetramer.[203,204] These interactions were hydrophobic in nature and were quite stable to disruption.[201] This contrasted with the relative weakness of interaction between the H2A-H2B dimers and the $(H3\text{-}H4)_2$ tetramer at physiological ionic strength.[201,205] Trypsin-modified nucleoprotein complexes have been subjected to DNase I digestion[206] and protein cross-

linking with dimethylsuberimidate.[207] It is has been concluded that the trypsin-limit peptides of the core histones retain both the structural elements necessary for the proper protein-protein associations and the ability to organize the DNA into a nucleoprotein complex which resembled the chromatin core particle. A series of recent studies by Böhm and co-workers[208-210] have defined the limit-digestion products of the core histones when chicken erythrocyte nuclei were extensively digested with trypsin. The limit peptides of the core histones were as follows: residues 12 to 118 for H2A, 27 to 129 for H3, 18 to 102 and 20 to 102 for H4, and 21 to 125 and 24 to 125 for H2B. These trypsin limit-digestion polypeptides contain those regions of each histone that were found by Bradbury and co-workers[203-204] to be essential for pairwise interactions. Notably, although each of the core histones is cleaved on its amino-terminal side, only H2A and H4 were found to be cleaved at the carboxyl terminus. In the case of chromatin, there are several lines of evidence that the carboxyl–terminal region of H2A might normally be accessible to enzymatic modification(s). Wu et al.[211] observed that the ubiquitin moiety, which is attached at residue 119 of histone 2A,[212] is in rapid equilibrium with the pool of free ubiquitin in both dividing and nondividing cells. In another study,[213] it was observed that although the amino-terminal domain of H2A is trypsin sensitive in all the conditions tested, the carboxyl–terminal domain of H2A is protected from the action of trypsin when organized in the core histone octamer in solutions of high ionic strength. This region of H2A became exposed to trypsin when the conditions were adjusted to reduce the association of the $(H3\text{-}H4)_2$ tetramer and the H2A-H2B dimers in the octameric complex. When the substrate for trypsin cleavage was the H2A-H2B dimer, or several forms of chromatin or nuclei, there was an ordered cleavage of the amino terminus of H2A followed by cleavage of its carboxyl–terminal region.[202] H2A-H2B dimers from which the carboxyl–terminal 15 amino acids of H2A had been specifically removed had a significantly reduced association with the $(H3\text{-}H4)_2$ tetamer.[214] From these studies came the suggestion that the carboxyl terminus of H2A is somehow involved in the formation of the dimer-tetramer contact interface in the octamer.

If the amino-terminal regions of the core histones are not necessary for histone-histone interactions or nucleosome stability, what then is their role? Azorin et al.[215] have suggested that the amino termini of the core histones are bound to the linker DNA between nucleosome cores. McGhee et al.[216] suggested that the histone amino termini of one nucleosome may interact with the DNA of another nucleosome. Allan et al.[217] conducted a series of experiments to assay the role of core histone amino termini in the higher order structure of chromatin. By a variety of techniques, they found that polynucleosomes containing core histones which had lost their amino termini by the action of trypsin were unable to fold properly into higher order solenoidal structures as were the control unproteolyzed polynucleosomes. Manipulation of the environment and the amount of histone H1 reconstituted with these proteolyzed polynucleosomes was without effect. However, if a 94-residue polypeptide from the highly basic, unstructured carboxyl terminus of calf thymus histone H1 (CH1) was reconstituted to the proteolyzed polynucleosomes prior to the addition of H1, these complexes exhibited the same higher order structural properties as the control polynucleosomes. Both CH1 and H1 were reconstituted at 2 mol equivalents per nucleosome. This would mean that the CH1 component would contribute two polypeptides with a total of 188 amino acids. From the studies of Böhm et al.,[208-210] it can be calculated that each nucleosome in the trypsinized polynucleosome chain would have lost a total of 148 to 158 amino acids from the amino termini and a total of 34 amino acids from the carboxyl termini of the 8 core histones. These two populations of polypeptides contain not only a similar number of amino acids, but also a similar proportion of basic amino acids (42% basicity for CH1 and 37% for the trypsin-sensitive histone tails). The authors suggest that the functional replacement of the trypsin-sensitive core histone tails with CH1 underlies the low specificity of these tails in the stabilization of the solenoidal chromatin structure. They also

suggest that the trypsin sensitive and resistant domains of the core histones need not be covalently linked. Testing a variety of polypeptide chains would help define more clearly the function of the trypsin-sensitive tails, for instance, in the simplest case substitution of polylysine for CH1. It is interesting to speculate that the more basic, unstructured terminal regions of the core histones may not only provide some electrostatic shielding of the DNA, but may also mediate the proper binding of histone H1 to the nucleosomal chain.

Grunstein and co-workers[144] have genetically manipulated certain strains of yeast so as to produce mutations in histone H2B. Mutations which deleted a large number of amino acids within the unstructured amino terminus of H2B had no apparent effect on the synthesis or function of this histone in vivo, or on the phenotype of the cells. However, mutations which caused not only deletion within the unstructured amino-terminal region, but also in the hydrophobic structured core of this protein, were found to be lethal. Yeast appears to lack histone H1,[218] and the mechanism of regulation of its higher order chromatin structure is unclear. Since trypsin removal of the accessible amino termini of the core histones has very little effect on the primary nucleosomal core structure, it is additionally apparent that in yeast the loss of a portion of the more basic, unstructured terminal regions of the histones is without any obvious effect on chromosomal structure and function.

B. Physical Studies: Diffraction Studies on Nucleosome Organization

Neutron scattering used with the technique of varied contrast has shown that the nucleosome core particle in solution has a hydrophobic histone core around which 1.7 turns of DNA are wrapped with a pitch of between 3.0 and 3.5 nm.[219-220] Bentley et al.[221] arrived at a similar conclusion (1.8 turns and a pitch of 2.75 nm) using the same techniques to analyze crystals of intact nucleosome core particles. Although the data indicate that most of the histone is located in the hydrophobic protein core, approximately 25% of the protein is located external to this core, a value quite similar to the percent of trypsin-sensitive residues in the histones. NMR studies of nucleosome core particles have shown that while the more central, globular regions of the histone polypeptides interact with the DNA in the particle, the amino termini of H2A and H2B are free and mobile, and the amino termini of H3 and H4 are only weakly bound, being dissociated in 0.6 *M* NaCl.[222] In a related observation, Palter and Alberts[223] found that the salt-extraction properties of histones from DNA were not changed by the trypsin removal of their amino-terminal regions.

The most detailed information on histone-histone and histone-DNA interactions is presently being determined by X-ray crystallography. Richmond et al.[224] have reported a 7 Å-resolution structure of the nucleosome core particle. The notable results of their crystallographic analyses were that the DNA does not bend smoothly into a superhelical path, but has several areas of tight bending, or even possibly kinking, near regions of substantial contact with histones H3 and H4. The location of the regions of electron density indicative of protein suggested that the core of the histone complex is located within the coils of the DNA superhelix. Heavy metal derivatization of histone H3 allowed its suspected location within the core particle to be confirmed. Assignment of regions of density within and along the helical ramp of DNA to the other histones was achieved by utilization of earlier data obtained by mapping the order of histones along the nucleosomal DNA by chemical cross-linking and DNA cleavage[225-226] and the information on histone-histone contacts as determined by protein cross-linking (reviewed in Reference 7). One of the H2A-H2B dimers within each unit (core particle) was found to interact with a core particle of an adjacent unit thereby putting the two dimers into different environments.

Burlingame et al.[227,228] have determined a 3.3 Å-resolution structure for the native core histone octamer. Their data obtained from crystals of the core histone octamer have shown that the octamer has perfect twofold symmetry. The degree of resolution obtainable at 3.3 Å should allow the determination of the position of the individual amino acids along

the backbone of each histone polypeptide within the core histone octamer. The combined effort of these studies should give considerable insight into the precise structure of the histones in chromatin. This can hopefully lead to an analysis of the structural and functional roles of variations in the histone primary sequence as well as the effects of the different types of post-translational modifications of histones.

C. Nucleosome Assembly In Vitro

A central role for the $(H3\text{-}H4)_2$ tetramer in the organization of the nucleosome core was discovered in 1976 when Camerini-Otero et al.[229] reconstituted by salt gradient dialysis almost all possible combinations of homogeneous histone fractions onto DNA and found that only several such combinations of histones were capable of organizing the DNA similarly to the way it is organized in chromatin. Analysis of the kinetics of micrococcal nuclease digestion of (H3 + H4)/DNA reconstitutes allowed them to conclude that the (H3 + H4) complex alone can organize the DNA into protected segments. A portion of the more nuclease-resistant DNA fragments were equivalent in length to what had previously been shown to be protected in the nucleosome core. Reports from two other laboratories indicated that it is possible for either one or two $(H3\text{-}H4)_2$ tetramers to interact in vitro with nucleosome core length DNA.[230,231] One major product of nuclease digestion of those reconstitutes was a complex which contains one tetramer with a DNA fragment of about 70 base pairs long.[231] Sollner-Webb et al.[181] found that H2A and H2B act together to modify the (H3 + H4)/DNA complex creating a structure which is recognized by a number of structural probes as being identical to the nucleosome core.

Stein[232] developed a salt-jump histone-DNA reconstitution technique which permitted analysis of the reassociation kinetics of the core histone complex with nucleosome core-length DNA. He found that approximately 75% of the DNA was immediately assembled into nucleosome core particle structures during and shortly after rapid reduction of the salt concentration from 2.5 to 0.6 *M* NaCl. The remainder of the DNA was more slowly assembled into core particle-like structures. It appears that about a third of the rapidly formed complexes contain an extra histone octamer and with time new core complexes are formed as these excess octamers are transferred to free core DNA. Daban and Cantor[233] extended these results with their study of the assembly of core histones onto core particle length DNA by the salt-jump technique[232] in which the concentration of salt was rapidly dropped from 2 to 0.2 *M*. They found that the newly formed (H3 + H4)/DNA complexes could be matured into native core particles if H2A and H2B were added during the next few minutes. If, however, 1 hr transpired, the (H3 + H4)/DNA complexes could not be matured by the addition of H2A and H2B. They concluded that in the absence of H2A and H2B the (H3 + H4)/DNA complex rearranges into a "dead-end" complex which cannot be subsequently matured by the addition of H2A and H2B into a properly folded nucleosome.

Like the studies discussed above, a great proportion of the in vitro studies of nucleosome core reconstitution have brought purified histones and DNA together in high salt concentrations and allowed reassociation of these components to occur as the salt concentration is reduced to much lower levels. A number of more recent studies have shown that it is possible to assemble these components into a proper nucleosome core structure at physiological ionic strength. Stein et al.[234] were able to reconstitute nucleosome core particles from core histones and core-length DNA (145 base pairs) by mixing them in 0.2 *M* NaCl at pH 8 and incubating for 16 hr at 37°C. Although an initial precipitate formed, during the incubation period a redistribution of the components brought about proper nucleosome core formation. Laskey et al.[235-237] studied the assembly of nucleosome cores in a cell-free system from the eggs of *Xenopus laevis* and by fractionation of the extract were able to identify the assembly activity associated with an acidic thermostable protein. The purified assembly factor protein, called nucloplasmin, is a pentamer of 29,000 dalton subunits, has approximately 30% acidic amino

acids, is phosphorylated, has an isoelectric point of 5, and is the most abundant protein in the *Xenopus oocyte* nucleus (reviewed in Reference 238). Although nucleoplasmin does not bind to DNA or chromatin, it has been found to bind histones in vitro. The protein has been shown to be localized at high concentrations in the nuclei of a wide variety of vertebrate cells.[239-240] Other analogous assembly systems have been described. The topoisomerase I of *Drosophila* was thought to be a physiological chromatin assembly factor but it was later determined that the assembly activity was actually a function of contaminating high molecular weight RNA.[241] Stein et al.[234] found that the acidic polypeptides, polyglutamic acid and polyaspartic acid, could both stabilize the association of the core histone octamer and facilitate nucleosome core assembly at physiological ionic strengths. In another study, histones were very slowly added to excess DNA at physiological ionic strength in the supposed absence of any assembly factors.[242] However, the bulk of the analysis in this study was done on complexes which were allowed to reconstitute in the presence of a high concentration of bovine serum albumin, a protein which, like nucleoplasmin, has a pI of 5.0. In a very recent study, rat liver HMG-1 was suggested to be a physiological assembly factor.[243] The carboxyl-terminal region of HMG-1 has a high proportion of acidic amino acids and for this reason may be able to mimic in vitro the physiological nucleosome assembly factors. In addition, HMG-1 has been found to bind preferentially to single-stranded rather than double-stranded DNA and to be capable of partial unwinding of the DNA helix. For optimal nucleosome assembly a 2:1 HMG-1:histone weight ratio was required. It is possible that at localized regions within the chromatin HMG-1 may function in the modulation of core histone or even histone H1 binding.

Thus nuclear assembly factors and functionally similar acidic polypeptides (as well as the high molecular weight RNA) may facilitate specific histone associations and core nucleosome assembly by providing a soluble system in which both the electrostatic repulsion of similarly charged histones and the electrostatic attraction of the differently charged histones and DNA are neutralized.

IV. POSTSYNTHETIC MODIFICATIONS OF HISTONES

In addition to the heterogeneity of their primary sequences, histones are also extensively modified. Various aspects of histone modifications have been recently discussed in a series of review articles and books.[14,125,244-257] Therefore, citations of earlier papers will be limited to the first report of a new kind of modification, its site or enzymology. More attention will be paid to some of those modifications that have not been so widely discussed than to the more commonly studied modifications such as acetylation and phosphorylation that have recently been extensively reviewed. The available data on how, where, and which histones undergo post-translational modifications are derived from four different kinds of experiments. The first group includes experiments performed in vivo in living organisms or cell cultures, sometimes with conditions altered to optimize for a particular modification.

The second group includes experiments performed on isolated nuclei. It is assumed that in such experiments most of the original structure of chromatin remains unchanged, and that differences may be studied more quantitatively because concentrations and conditions can be controlled exogenously. However, changes in the composition of the nucleoplasm may lead to changes in the localization of enzymes and/or the accessibility of substrates influencing reaction kinetics. A third group represents experiments in vitro on isolated chromatin fragments.

Finally, the fourth group of experiments consists of enzymatic reactions on purified histone fractions or even variants. With in vitro reactions, the reactivity of a purified macromolecule toward a modifying enzyme may be limited to a single site or, depending on the degree of specificity of the enzyme, may include several sites. Changes in the medium influence the shape and charge of the macromolecular substrate as well as the catalytic properties of the

enzyme, and these influences may not be the same at each site. With macromolecules, such as histones, which become part of higher order structures, the relevance of the reactions studied in the fourth group of experiments to those taking place in vivo depends on the relative accessibility of the substrate in vitro vs. in vivo. Some modifications reported in vitro, such as the phosphorylation of histone H1 at Ser_{103},[250] have never been detected in vivo. In such cases the reason could be that in vivo the active enzyme is not present at the site or the site is completely inaccessible; however, it may also be that the reaction does occur, but at a low level, for a brief time, or in a physiological state of the cell not studied.

A. Types of Modifications

Of a large selection of post-translational modifications found in different proteins,[258,259] only a limited number have been proven to exist in histones. The following kinds of modifications will be subsequently described: alkylation, acetylation, phosphorylation, oxidation-reduction, poly(ADP-ribosyl)ation, ubiquitination, and hydrolysis of peptide bonds.

Table 1 lists which histones are known to be modified and at which sequence position. Histone sequences have been accumulating recently at an increased rate but many of them were obtained indirectly from sequencing histone genes. Protein sequences determined indirectly contain no information about sites of post-translational modifications. The information in Table 1 is limited to discoveries of new sites in new histone species. Subsequent papers stating that a previously described modification has been found in, or is absent from, another species and/or tissue have not been included. A variant modification will be listed only if its position cannot be identified with one already known in other variants.

1. Alkylation

a. N-Methylation

Methylation of the amino terminal to form *N,N,N*-trimethylalanine[260] has been reported in *Tetrahymena*. Methylation of internal lysines to form N^6-methyllysine,[296] N^6-dimethyllysine,[297] and N^6-trimethyllysine[261] has been reported for all four core histones; in H3 and H4 the site has been determined in some cases (Table 1). The precise estimation of the degree of methylation of individual positions in proteins is difficult because subsequent methylation causes little difference in the properties used for the separation of these derivatives. Thus, di- and trimethyllysines, overlooked in an earlier study,[296] were found later after optimizing the conditions used for their separation in the amino acid analyzer.[261,297] The degree of methylation is not shown in Table 1.

A group of enzymes catalyzing methylation of lysines at N^6 is known.[298] They differ from each other by showing high specificity toward the protein as a substrate and toward the particular lysyl residue within the polypeptide chain. There are no data available yet comparing the characteristics of methylases for a given lysine in a given histone from different tissues or species or providing insight into the factors influencing the number of methyl residues introduced into a given lysine. However, the degree of methylation is probably not random because the ratios of the average methyl content are characteristic for particular proteins and tissues.[263] The assumption that histone methylation is totally irreversible had to be abandoned since a mammalian N^6 alkyllysinase, catalyzing the removal of lysine-bound methyl groups from histone, has been discovered.[264,298] Its activity varies greatly from one tissue and species to another.

Methylation of the guanidine nitrogen of arginine to form *N*-methylarginine,[299] *N*-(symmetric) dimethylarginine, and *N*-(asymmetric) dimethylarginine[299,300] has been reported, as has methylation of the imidazole nitrogen in histidine to form N^1-methylhistidine.[301] There is indirect evidence for arginine methylation in all the histone species (Table 1).[267,269,299,300] The enzyme catalyzing the methylation of arginyl residues has been isolated from several sources. It catalyzes the formation of all three methylated derivatives known; namely, N^G-

Table 1
POSITIONS OF THE VARIOUS MODIFICATIONS OF THE FIVE CLASSES OF HISTONES

	H2A		H2B	
Modification	**Site**	**Ref.**	**Site**	**Ref.**
Methylation[a]				
N-terminal			A_1[b]	260
N-side chain	(K_+) R_+	261—265	(K_+)	261—265
		266—269	(R_+)	266, 268, 269
O-side chain	(D/E_-)	275, 276	(D/E_-)	275, 276
Acetylation				
N-terminal	S_1	277		
	M_1	105		
N-side chain	K_5	278, 279	K_5	278
	K_{12}	279	K_{10}	278
			K_{13}	278
			K_{18}	278
Phosphorylation				
O-side chain	S_1	281		
	S_{122}[c,d]	279	S_{32}	282
	S_{124}[c]	279	S_{36}	282
	S_{128}[d]	279		
	S_{129}[c]	279		
N-side chain				
ADP-ribosylation	(?)	290	E_2	290—292
Ubiquitination	K_{119}	212	?	295

	H3		H4	
Modification	**Site**	**Ref.**	**Site**	**Ref.**
Methylation[a]				
N-terminal				
N-side chain	K_4	260, 261	K_{20}	28, 31, 265, 271, 274
	K_9	265, 270, 271	K_{79}	280
	K_{27}	265, 270—273	R_+	267
	K_{36}	273		
	(R_+)	266, 268, 269		
O-side chain	(D/E_-)	275, 276	(D/E_-)	275, 276
Acetylation				
N-terminal			S_1	28
N-side chain	K_9	278	K_5	278
	K_{14}	278	K_8	278
	K_{18}	278	K_{12}	278
	K_{23}	278	K_{16}	278
Phosphorylation				
O-side chain	T_3	283, 284	S_1	28
	?	285, 286	S_4	287
			S_{47}	287
N-side chain			H_{18}	244, 288
			H_{75}	244, 288
ADP-ribosylation	(?)	290	(?)	290

Table 1 (continued)
POSITIONS OF THE VARIOUS MODIFICATIONS OF THE FIVE CLASSES OF HISTONES

	H1/H5			
Modification	**Site**	**Ref.**		
Methylation				
N-terminal				
N-side chain	H_1	266		
	(K_1)	261, 265		
	(R_1)	320, 322, 323		
O-side chain	(D/E_1)	275, 276		
Acetylation				
N-terminal	S_1	124		
	A_1	136		
	(P_1)	246[g]		
N-side chain				
Phosphorylation				
O-side chain	T_2	244	S_3[c]	251
	S_{15} or $_{16}$	244, 250	S_7[c]	251
	T_{19} or $_{20}$	250	S_{104}[c]	251
	S_{37} or $_{38}$	244, 250	S_{117}[c]	251
	S_{103} or $_{105}$[f]	244, 250	S_{148}[c]	251
	S_{114}	244		
	T_{136}	244, 250		
	S_{145}	250		
	T_{153}	244, 250		
	S_{161} or $_{173}$	250		
	S_{180} or $_{182}$	244, 250		
N-side chain	K_1	244, 289		
ADP-ribosylation	E_2	293, 294		
	E_{14}	294		
	(E_{116})	293, 294		
	K_{213}	294		

Note: References are noted only for the primary discovery. The sequence positions used in this table are the ones originally given by the cited authors. In some cases they may differ from the positions in other variants due to slight differences in sequence. Modifications suggested from indirect evidence only are in parentheses. A question mark is used when the precise site of the modification is not known.

[a] Three levels of methylation of lysine and two of arginine are possible. In most cases, all forms are present but in varying quantities (not shown in Table).
[b] In *Tetrahymena* only, trimethylated.
[c] H2A(1).
[d] H2A(2).
[e] H5 as substrate in vitro.
[f] In vitro only.
[g] Not mentioned in Reference 137.

mono-, N^G, $N^{G\prime}$-di(symmetric) and N^G, N^G-di(asymmetric) methylarginine at constant ratios for a given substrate.[300] Species, tissue, and substrate specificities remain unknown. No data are available on arginine demethylation.

b. O-Methylation

Based on the susceptibility of labeled methyl derivatives to alkaline hydrolysis, it seems that several histone species can be methylated at aspartic and glutamic acid residues when incubated in vitro with partially purified preparation of Methylase II.[275,276,298] Because the

samples of commercial histones used in these experiments were heterogeneous and nonreproducible and the separation techniques applied in earlier works did not prevent cross-contamination among histone species, it is not certain which histones were in fact methylated. O^4-methylaspartic acid and O^5-methylglutamic acid[275,276] have never been isolated from histones.

The documentation suggesting *O*-methylation of histones in vivo is not fully convincing[275] either, considering the low level of labeling and the presence of many nonhistone proteins in the poorly defined endogenous substrate used. The detection techniques, autoradiography or fluorography, were sensitive enough so that a minor contaminant co-migrating with a histone fraction in their gels or columns may easily have suggested labeling of the main fraction. As stated by Kim,[276] no method is available to quantitate the methyl esters due to their instability in both mild acidic and alkaline media. Therefore, this modification requires new improved techniques to identify if histones are being modified, and if so, at which positions.

Enzymes of eucaryotic origin which methylate free carboxyl groups of histones in vitro have been isolated and characterized from a variety of mammalian tissues[276] and from wheat germ.[275]

c. Chemical Methylation

If cells are treated with potent alkylating agents, some unusual derivatives of histones may occur due to chemical alkylation. In vivo administration of dimethylnitrosamine[302,303] leads to the formation of N^3-methylhistidine and *S*-methylcysteine.[302] Administration of dimethylnitrosourea[304] leads to the formation of as yet unidentified methylated derivatives of histones. If diethylnitrosamine is used instead of its methyl homologue, corresponding ethylated substitutes are formed.[302]

2. Acetylation

Of the limited selection of amino-acyl substitutions found in proteins,[258,259] amino-acetylation is the only one present in histones. Two kinds of acetylation should be differentiated. The first is amino-terminal acetylation, which is irreversible. The acetylated amino-terminal amino acids that have been found in different histones are *N*-acetylserine[277] (most common), *N*-acetylmethionine,[105] *N*-acetylalanine,[136] and possibly *N*-acetylproline.[137,246] Based on the observation of Lebherz et al.,[305] Wold suggested that acetylation of amino-terminal amino acids, instead of being a specific modification of selected amino terminals, represents a general mechanism of amino-terminal processing which would involve a transacetylase followed by a peptidyl-exohydrolase.[306] Wold's model, suggested for most soluble cytoplasmic proteins, may also be valid for histones, where H2A, H4, and H1 usually have acetylated amino terminals, while H2B and H3 do not.

The second kind of acetylation, side-chain acetylation,[307] is one of the most ubiquitous and widely studied modifications of histones. It has also been extensively reviewed.[125,247-249,252,278] All four core histones in mammals are acetylated at multiple known lysines (Table 1), but the lysine-rich histone H1 does not seem to be acetylated at all (besides amino terminus). The mass pattern of histone modification seen on polyacrylamide gels is largely due to the acetylated forms. In spite of this, the functions of acetylation are not known. The effect of acetylation on histone interaction is discussed in Section III. The attempts to correlate certain forms of acetylation with transcriptional activity are discussed later in this section.

Several acetylases have been described and the results have been extensively reviewed recently.[247,252,278] Others are also being isolated and characterized.[308-310] The different members of this class of enzymes differ greatly in general properties including substrate specificity.

Lysine acetylation at N^6 is reversible, catalyzed by a group of highly active deacetylases.[311]

Sodium butyrate has been shown to strongly inhibit the deacetylase, shifting the equilibrium to the highly acetylated forms.[312] The labile equilibrium makes the evaluation of the level and degree of acetylation of histones in vivo more difficult. The fact that hyperacetylation of histone is not immediately lethal to cells suggests that the level of acetylation is related to the fine control of regulatory processes rather than to the fundamental stability of the nucleosome.

3. Phosphorylation

Of the several amino acids known to be modified by phosphorylation,[258,259] O^3-phosphoserine,[313,314] O^3-phosphothreonine,[314] N^6-phospholysine,[289,315] and N^3-phosphohistidine[288,289,315] have been found in histones (Table 1).

Phosphorylation is another widely studied modification of histones and the topic has also been extensively reviewed recently.[125,244,247-251,278] As will be discussed later, changes in the phosphorylation of certain histone moieties have been correlated with chromosome condensation at mitosis.

A series of protein kinases, for which histones are the optimal or exclusive substrates, have been isolated recently from various sources, some of them in homogeneous form. The name "histone kinase" is used for activities which use purified total histone or a purified histone fraction as substrate much more efficiently than any other protein tested. Histone kinases have been comprehensively described in a recent review by Matthews and Huebner.[244] They are classified according to their substrate specificity, localization, cyclic AMP dependence, and activity fluctuations during the cell cycle. Substrate specificity includes particular positions in the amino acid sequence susceptible to phosphorylation by the given enzyme.

The structural requirements for specificity in vivo are much more complicated. For example, the plasmocytoma histone H1 kinase purified from a microsomal fraction in the almost homogeneous state[316] uses purified H1 in vitro as a substrate but appears completely inactive toward H1 in chromatin or nucleosomes. In addition, the identical enzyme appears to be present in plasmocytoma chromatin,[316] and this enzyme is probably the same as the mitotic[317] and growth-associated histone kinases from several rapidly dividing cells.[244] The reactivity of purified H1, but not chromatin-bound H1, again demonstrates that simple substrate specificity, as evaluated on purified histones in vitro, cannot be directly applied to chromatin where the accessibility of the substrate is surely very different.

On the one hand, more and more new kinases are being purified and tested on isolated histones for specificity: a cAMP-independent kinase from bovine thymus (acting on Ser_{32}, but not on Ser_{36} of the H2B histone),[282] a cAMP-independent kinase from rat epididymis,[318] a Ca-dependent kinase from HeLa cells (acting on a serine residue within the trypsin-sensitive amino-terminal domain of H3),[285] a cAMP-independent endogeneous kinase from bovine thymus (acting on Thr_3 of both free and chromatin-bound histone H3, the former causing gradual enzyme inactivation),[283] a spermine-activated kinase from myotube-stage myoblasts,[286] cAMP-dependent kinases I and II from proliferating lymphocytes (acting on Ser_{47} (kinase I) and Ser_1 (kinase II) of histone H4),[287] and kinases phosphorylating histone H2A.[279,319,320]

On the other hand, very little can be said about the specificity requirements of these enzymes in vivo. Even though some of these enzymes appear active toward isolated chromatin or its subunits, these substrates give us no useful insight into the regulation of the modification process.

Even less is known about enzymes catalyzing *N*-phosphorylation of histones. Since the report in 1974 of the existence of two "P-N"-kinases from several mammalian tissues,[244] no further progress in isolation and characterization of this group of enzymes seems to have been reported.

Recently, partial characterization of several phosphohydrolases or phosphatases specific for histones has been reported.[321,322] These findings are in agreement with the well-known reversibility of histone phosphorylation.

4. Oxidation of Cysteine SH Groups

H3 is the only sulfhydryl-containing histone in most eucaryotic organisms, except echinoderms which also have a sulfhydryl-containing H4. The H3 variants of higher mammals contain one or two cysteine residues, both in the conserved globular domain at the positions 96 and 110. A comparison of the increasing number of sequences now available reveals that the H3s of most lower animals do not have a cysteine at position 96. Yeast H3 histone has no cysteine at all, the two positions being substituted by valine and alanine.

Except for some earlier observations concerning metaphase[323-326] chromatin, no post-translational modification of cysteine in histone H3 has been reported. However, at low ionic strength, the sulfhydryl groups at $cysteine_{110}$ of the two H3s in the same nucleosome are close[327] enough and accessible enough to oxidizing agents to be directly oxidized into an intermolecular S-S bridge.[328,329] The resulting H3 dimer can still be used for in vitro octamer assembly.[330] At physiological ionic strength, both sulfhydryl groups are buried inside of the globular folded part of the molecule and are thus inaccessible to even low molecular weight reagents.[331-334] However, there is evidence that they may become exposed in a more open structure in transcribing chromatin[335] (see Section VI). The cysteines have also been used in structural studies as spots of unique reactivity[336] for labeling nucleosomes in vitro.[328-335]

A series of mammalian enzymes involved in the formation and disruption of S-S bridges in proteins have been described: a bovine thioredoxin,[337,338] a rat protein disulfide reductase,[339] and a protein disulfide reductase from pea seeds.[340] Whether some of these activities are present in the nuclei has not been established. The proof of such activity in vivo, namely the formation of H3 dimers under conditions where artifacts could be ruled out, is lacking (see, however, Reference 325).

5. ADP-Ribosylation and Poly(ADP-Ribosyl)ation

This modification, detected by Nishizuka et al.,[341] is performed mainly by a single enzyme, poly(ADP-ribose)synthetase,[342] which is found associated with chromatin. The following adducts have been reported for histones treated in vitro: mono- and poly(ADP-ribosyl) 5-0 glutamic acid,[293] mono- and poly(ADP-ribosyl) 1-O lysine (carboxyl terminal),[294] and di-[poly(ADP-ribosyl)-H1][343] (see comments in References 291 and 344). The character of the bond existing at the other end of the approximately 15-unit-long poly(ADP-ribose) linker remains unknown, but there is evidence reported that the linker may join a glutamate residue within the amino-terminal domain of one H1 molecule with a position within the carboxyl-terminal domain of a second one.[344] The poly(ADP-ribose) polymer in vitro varies in length from a single residue up to tens of residues and contains both linear and branched chains,[345] but no data are available concerning the factors which influence the final length of the polymers and their branching. ADP-ribosylation of histones has not been found in vivo.

The poly(ADP-ribose)synthetase[342,346] is highly specific toward NAD, but appears much less so toward the second substrate. The same enzyme is supposed to introduce the first ADP-ribose molecule into the protein substrate (in several positions), attach subsequent residues in the elongation process, form branched structures to ADP-ribosylate itself, and form free poly(ADP-ribose) polymers. Both H2B at Glu_2 and H1 at Glu_2 and Glu_{14} as well as the carboxyl-terminal lysine of H1, all are substrates for the same enzyme.

The existence of bonds other than those listed above has been postulated based on observations of adducts resistant to neutral NH_2OH[291,345,347-349] and even to alkaline media.[347] Their nature remains unknown but several suggestions have been made. ADP-ribosyl trans-

ferases are cytoplasmic enzymes which transfer single residues of ADP-ribose to arginine or lysine residues of proteins. It was suggested that such a transferase may bind an ADP-ribosyl group to free cytoplasmic histone, and then a poly(ADP-ribosyl)polymerase would carry the elongation process in the nucleus.[349] One of the ADP-ribosyl transferases is reported to be found in the nucleus.[350]

Several other enzymes synthesizing and degrading poly(ADP-ribose) and mono(ADP-ribose) derivatives of histones have been described.[351]

Poly(ADP-ribose)glycohydrolase disrupts the bond between the two ribose residues connecting two subsequent ADP-ribose units in the polymer chain. It degrades chains of ADP-ribose residues except the last one, which is directly attached to the protein molecule.[352,353] (ADP-ribose) protein lyase removes the last ADP-ribose residue attached directly to the protein, by a nonhydrolytic mechanism.[351,354] Poly(ADP-ribosyl)protein pyrophosphatase hydrolyses phosphoanhydride bonds in poly(ADP-ribosyl) chains.[355]

6. Ubiquitination

Ubiquitin was found attached to Lys_{119} of H2A[212] in a very unusual branched chain protein (uH2A). Ubiquitin adducts have also been found for H2B (uH2B).[295] The attachment site was localized carboxyl terminal to the methionine residues of H2B, but the exact position at which ubiquitin binds to the H2B histone has not been defined.

Ubiquitination processes in the cytoplasm are involved in controlled proteolysis[257,356] and it has been suggested that ubiquitination of histones is part of the above mechanism. The hydrolase (isopeptidase), which degrades the pseudopeptide bonds, has been isolated, partially purified, and characterized.[357,358] The ubiquitin ligase system, composed of three enzymes and probably involved also in uH2A formation, has also been characterized.[356,359]

7. Enzymatic Hydrolysis of Specific Peptide Bonds

Histones are not generally synthesized as longer preproteins, but are released from the ribosome with their ultimate sequence. One known exception is a variant of *Tetrahymena* H3 which lacks a peptide six residues long from its amino terminal.[360] This variant is localized in only one of the two differentiated *Tetrahymena* nuclei. This localization is suggestive of a differentiated function but otherwise the significance of this finding is not yet apparent. The proteolytic activity involved in the specific post-translational proteolysis of the H3 histone from *Tetrahymena*[360,361] has not been characterized.

Many proteolytic activities appear to exist in the cell nucleus, some of them as chromatin constituents, and some have been shown to selectively degrade histones. Little is known about their localization, sequence specificity, and the subsequent steps of the selective degradation of histones.[362-364]

It should be mentioned that even the removal and total degradation of a histone from a nucleosome could be considered a reversible modification of that nucleosome just as, for example, acetylation or phosphorylation could and perhaps should be thought of as a modification of the nucleosome rather than solely of the histone molecule. Proteases highly specific for H1,[365-367] H3,[362] and H2A[368] have been found and it has been shown that H1 proteolysis may be correlated with the degradation of poly(ADP-ribose)polymerase,[367] which in turn seems to have a role in DNA repair processes.[369] One or more histones in a nucleosome could be degraded or removed intact. If removed intact, they could later be returned to that nucleosome, or newly synthesized histones could replace the removed ones. If, however, removal requires previous degradation,[257] only new molecules may be used for a regulatory process based on histone degradation and replacement; histone turnover is discussed in Section V.

B. Histone Modification and Functional State

A large number of publications have appeared recently supporting or denying correlations between histone modifications and functional states of interphase chromatin.[280,370-392] Attempted function-modification correlations include: protozoan conjugation,[361] senescence of cells,[310] various aspects of differentiation,[283,286,370-373] neoplastic cells treated in different ways,[292,369,374,375] spermatogenesis,[376-379] repair mechanisms,[292,369] morphological[382] and physicochemical[383] observations of changes induced by modification, correlations of histone modifications with HMG content,[384, 385] and correlations between two types of modifications.[367,384-386]

In the case of spermatogenesis, a high degree of H4 acetylation is correlated with the replacement of histones by protamines or protamine-like proteins.[271,272]

Many of the current studies on histone modification and transcription are based on selective degradation of chromatin followed by separation of soluble products. Selective solubilization is attributed to differences in accessibility of DNA phosphodiester bonds in "active" vs. "inactive", "replicating" vs. "nonreplicating", "satellite" vs. "nonsatellite", etc. regions of chromatin. One problem is that the chromatin preparations, from which histones are isolated, are certainly in some respects less heterogeneous than the initial total preparations, but upon closer study, these are found to be generally still very crude. A second problem is the alteration or destruction of the macromolecules during the isolation and separation process. In order to help preserve native structure, various compounds such as any of several proteolysis inhibitors, sulfhydryl compounds, or sodium butyrate (an inhibitor of very active deacetylases) are usually included during chromatin isolation. A third problem is that the modifications found represent an average for the material investigated and in many cases only a small fraction of any histone species is modified. Thus, using this method to attempt to establish a correlation between a particular kind of histone modification and a particular functional state of chromatin has not been very successful. Attempts to establish any sort of cause and effect relationship have been even less successful.

Two recent reports, both using the acellular slime mold, *Physarum polycephalum*, illustrate attempts to circumvent some of those problems. Waterborg and Matthews,[387] in a carefully performed study, compared histone acetylation patterns in *Physarum*. Rather than fractionate the chromatin, histones were extracted directly from plasmodia under conditions in which loss of modification was minimized. The investigators took advantage of the synchronous cell cycle to compare rates and levels of histone acetylation in nuclei from S phase and G2 phase. Using computer analysis of the data they were able to dissect the S-phase pattern into the sum of two other patterns, the G2 pattern and a replication pattern called S-specific pattern. In the S-specific pattern, short pulses of radioactive acetate labeled primarily monoacetylated H4 (b_1H4), while in the G2 pattern, mainly higher adducts were labeled (b_2, b_3, b_4H4). Because the G2 pattern was affected by inhibitors of transcription but not replication or translation, the authors suggested that this supported a correlation between higher acetylated forms of H4 and transcription.

In a different type of study, but also on *Physarum*, Loidl et al.[388] treated plasmodia with butyrate to hyperacetylate the histones, then assayed the template activity of the chromatin in vitro with exogeneous RNA polymerases. They reported no difference in the template activity, and suggested that there was no correlation between histone acetylation and transcription.

These two studies mirror the state of this field. Using different techniques in the same organism, they reached opposite conclusions. Both studies were indirect and both were in some ways dependent on inhibitors for their correlation or lack of correlation between acetylation and transcription. In *Physarum*, it may be possible to study this relationship more directly by analyzing histone modification in the lexosomes (see Section VI) from active ribosomal genes.

In two rather special cases, investigators have been able to make some more convincing correlations between histone modification and chromatin function.

1. Free or Unbound Histones

It is well established that *N*-terminal methionine is removed from growing polypeptide chains during translation,[393] and it is very likely that the amino-terminal acetylation and methylation happen at this stage. In addition to being locked at the amino terminal, newly synthesized H4 isolated from the cytoplasm is doubly modified. Ruiz-Carrillo et al.[389] provided evidence that these H4 modifications were an acetylation and a phosphorylation. Other work has supported this result. Wu et al.[390] showed that H4 synthesized in G1 and G0 cells when DNA replication was absent was also doubly modified. They further showed that the nascent H4 found in mononucleosomes was doubly modified, but that these modifications were lost within 1 hr. The same process seems to takes place in S phase although much more rapidly. The change in the modification of the nascent H4 in nucleosomes may be involved with the process of nucleosome maturation. High levels of dimodified H4 are also found during the cleavage of sea urchin embryos.[392]

2. Chromosome Condensation and Mitosis

The whole genome participates in the process of chromosome condensation and mitosis; thus, in synchonized cell populations, it is possible to make some definite correlations. The most convincing evidence of such a correlation is the coupling between the phosphorylation of particular H1 sites and chromosome condensation during G2 and metaphase [394] (for recent reviews, see References 244, 250, and 251). An M(mitosis)-kinase, active during late G2[316,391,394] and distinct from other H1 kinases which are active throughout the cell cycle,[250] phosphorylates side-chain hydroxyls in the sequence (lys)-ser/thr-pro. At least one site in the amino-terminal domain and at least three sites in the carboxyl-terminal domain of all H1 molecules are phosphorylated. Other changes in histone modification are reported to take place at the time. All H3 molecules become monophosphorylated.[391] H2A and H2B lose the ubiquitin adducts.[395] There is also an increase the poly(ADP-ribose) content of nuclear proteins found in late G2 and M.[396]

However, there have been reports from other laboratories that do not fully support these findings, either due to differences in the species or tissue, or to differences in procedure. Some investigators have reported H3 phosphorylation at different times in the cell cycle and have suggested other correlations.[283-286] The degree of ubiquitination may not be complete and may be the effect of impairment of synthesis rather than of increased degradation.[257,397] Because of the limitations in synchronizing mammalian cells, it is generally necessary to isolate metaphase chromosomes in order to show a disappearance of uH2A and uH2B.[211] The level of poly(ADP-ribosyl)ation distinctly increases during G_2 and mitosis,[396] but other investigators also find maxima at other times.[398] The change in the level of poly(ADP-ribosyl)ation is not as striking as the increase in H1 phosphorylation. Thus, there is still some disagreement concerning the universality of a correlation between these modifications and mitosis.

In summary, histones are modified in many ways, but how these modifications are involved in the functioning of chromatin is still unknown. At best, correlations between histone modification and a functional state have been obtained in certain favorable situations. However, convincing correlations between any kind of histone modification and gene transcription remain elusive.

Some of the modifications described in this section are considered to be irreversible at the chromatin level. These irreversible modifications, such as methylation of arginine and histidine, or hydrolysis of specific peptide bonds, increase the diversity of the elements present in chromatin. The other modifications are in general reversible; these presumably

help provide flexibility in the regulation of changes that take place on relatively short time scales.

V. HISTONE SYNTHESIS AND CHROMATIN ASSEMBLY

The life of a somatic cell can be thought of as having two states, proliferation and nonproliferation. This later state is also commonly called quiescence but this is somewhat of a misnomer, since a great amount of cellular activity may be going on in the nonproliferating cell. In multicellular organisms, most cells are not actively proliferating. The proliferation state is composed of the cell cycle,[399] which in turn is composed of four phases, a cytologically distinct phase M (mitosis), a biochemically distinct phase S (DNA synthesis), and two gaps, G1 (between M and S), and G2 (between S and M). More recently, the cell cycle has also been thought of as the coordination of two cycles,[400] a nuclear cycle encompassing S, G2, and M, and the growth cycle, which has at present no distinguishable phases. In this view, G1 is that part of the growth cycle left over after the nuclear cycle is finished. It has been widely debated in the literature whether the nonproliferation state, G0, is an indefinitely long G1 or a separate state.

A. Histone Synthesis

As already described in some detail, histones are a group of small proteins, present in the chromatin of all eucaryotic nuclei. In vertebrates, the genes usually,[20,42,76,401] but not always,[57] lack introns and are clustered but not reiterated.[20,42,76,401] This arrangement differs from that of the histone genes active during the cleavage of certain zygotes, but not from the genes active during the later development of these same organisms.[20] Histone mRNA sediments at 7-9S and in general is not polyadenylated, although some, including yeast histone mRNAs and chicken H2A.Z mRNA, are polyadenylated.[102,402] Histone proteins are synthesized on small cytoplasmic polysomes (for review see Reference 403) and, by uncharacterized mechanisms which may involve "molecular chaperones" like nucleoplasmin,[235-240] migrate into the nucleus where they become incorporated as nucleosomes.

By the late 1960s it was thought that virtually all histone synthesis took place during the S phase of the nuclear cycle,[403] and was therefore linked or at least temporally coordinated with DNA replication. More recently, it has been established that significant histone synthesis takes place both in the non-S phases of the cell cycle,[404] and in the nonproliferation state G0.[405] These recent results were convincing for two reasons. First and most importantly there were qualitative differences in the variant patterns of histone synthesis in G1, G0, and S (Table 2). Thus the histone synthesis seen in G1 or G0 cells could not be attributed to contamination of these cells with S-phase cells. Second, this histone synthesis was insensitive to inhibitors of replication. When the fate of these histones was also studied, it was demonstrated that histones synthesized in G1 and G0 were incorporated into nucleosomes in the absence of DNA replication.[390] Thus, although most histone synthesis does occur during S phase, significant histone synthesis and incorporation into chromatin also occurs outside of S phase.

1. Histone Synthesis During Replication

Most of the histone synthesis that takes place during replication is coordinated with DNA synthesis. This coordination is functional and not just temporal because histone synthesis is inhibited when DNA synthesis is inhibited during S phase, but resumes when DNA synthesis is allowed to resume. However, some histone synthesis persists even when DNA synthesis is inhibited; this histone synthesis has the same variant pattern as that found in G1 and G2 cells. This synthesis, named basal synthesis,[404] continues throughout the cell cycle. During S phase, replication-linked histone synthesis from a presumably different set of genes is added to the basal synthesis.

Table 2
PATTERNS OF HISTONE SYNTHESIS IN CHO CELLS IN VARIOUS STATES

	Relative histone synthesis	H3 variants synthesized			H3 ratio 1 + 2 / 3	H2A variants synthesized				H2A ratio 1 + 2 / X + Z
		.1	.2	.3		.1	.2	.X	.Z	
S phase	(100)	+	+	+	2.5	+	+	+	+	3.5
G1 phase	5	−	−	+	0.3	−	−	+	+	0.4
G0 state	6	−	−	+	0.3	+	+	+	+	3.9

Note: Synthesis was determined by labeling cells in the indicated phase or state with radioactive lysine or arginine. The ratios of synthesis were determined by excising and digesting the stained protein islands of the indicated variants from two-dimensional polyacrylamide gels, followed by scintillation counting of the digest. See References 404 and 405 for details.

In a particularly important contribution to this field, Butler and Mueller[406] showed that when DNA synthesis was inhibited, histone mRNA levels decreased, and this decrease resulted in the inhibition of histone protein synthesis. They also found that inhibition of protein synthesis simultaneously with DNA synthesis prevented the decrease of histone mRNA levels. When histone gene probes became available, these questions about histone mRNA levels, synthesis, and stability were reinvestigated. Histone mRNA levels were found to be elevated during S phase and very low outside of S phase, including G1 and G0.[407-412] When inhibitors of DNA synthesis were added to cells, the material hybridizing to histone gene probes rapidly decreased to a low basal level.[413-418] Thus, the histone mRNA present during S phase did not just become untranslatable, but was destroyed. The low basal level of hybridizable material presumably is due to the transcripts for basal variants, which, at least in some cases, cross hybridize to the gene probes used. Using a gene for a basal variant, H3.3, and S1 nuclease to degrade poorly matched hybrids, Sittman et al.[413] showed that the level of H3.3 mRNA did not decrease when cells were treated with inhibitors of DNA synthesis.

Butler and Mueller[406] had also shown that simultaneous treatment of cells with cycloheximide and hydroxyurea prevented the decrease in translatable mRNA seen with hydroxyurea treatment alone. Stimac et al.,[419,420] assaying mRNA levels by hybridization to histone gene probes, substantiated those results and further showed that treatment of cells with cycloheximide alone led to increased levels of histone mRNA. These results have been extended to include the mRNAs for all five histones, as well as several inhibitors of protein and DNA synthesis.[414,416,419] In addition, several groups have shown that even after histone mRNA levels have been depressed by treatment with an inhibitor of DNA synthesis, subsequent addition of an inhibitor of protein synthesis allows histone mRNA levels to rise, perhaps even higher than in the untreated controls.[414,419]

Wu et al.[404,405,421] and Sariban et al.[422] have investigated the patterns of histone protein synthesis after various treatments with inhibitors of protein and DNA synthesis. Protein synthesis and histone synthesis could be analyzed even while partially inhibited by treatment of cells with cycloheximide or puromycin. Treatment with inhibitors of DNA synthesis during replication altered the pattern of histone variant synthesis to the one seen in G1 cells.[404] Inhibition of protein synthesis with puromycin or cycloheximide simultaneously with the inhibition of DNA synthesis prevented this alteration in the pattern of histone-variant synthesis even though histone synthesis was quantitatively decreased. Inhibition of protein synthesis in cells in which DNA synthesis had already been inhibited resulted in a return to an S-phase pattern. Inhibition of protein synthesis alone led to an increase in the

proportion of S-phase variant synthesis. The changes in the level of histone mRNA were quickly reflected in the histone-variant synthesis pattern indicating that the histone mRNA is immediately translated.[421,422]

2. Models

The rather complex interactions described above are not adequately explained by the existing models. In one of the earliest and most complete of these models, Butler and Mueller[406] proposed that the level of translatable histone mRNA was in some way determined by the concentration of free histone or histone not bound to chromatin, and they found evidence for a small free histone pool in the cytoplasm. According to their model, inhibition of DNA synthesis blocked the utilization of histone from the pool; as the free histone became more concentrated due to continued protein synthesis, it inhibited the expression of its own mRNAs and genes. Simultaneous inhibition of protein synthesis prevented the rise in the concentration of free histone, thus the feedback controls were not greatly altered and translatable histone mRNA levels did not change very much. However, their model predicted that once DNA synthesis was inhibited and translatable histone mRNA levels had decreased, those levels could not rise again until DNA synthesis was allowed to resume since the elevated level of free histone had no way to decrease when replication was blocked. This prediction was contraindicated by the experimental results discussed above. After histone mRNA levels had decreased due to the inhibition of DNA synthesis, they increased when protein synthesis was inhibited during the continued inhibition of DNA synthesis.[414,419]

The inadequacy of the autoregulation model as originally proposed[406] centers primarily on the dependence of changes in the level of free histone on changes in the rate of DNA synthesis. However, it has been shown that in cells in G1 or G0, when there is no DNA synthesis, histones are synthesized and incorporated into chromatin as nucleosomes; the rates are slower than during replication, but fast enough so that the free histone pool could reflect changes in protein synthesis.[390] Thus, when an inhibitor of protein synthesis is subsequently added after an inhibitor of DNA synthesis had already acted, the production of histone would be further inhibited, and the level would fall. By autoregulation, the histone mRNA level would rise, even though DNA synthesis had been inhibited throughout. In the same way, the elevation of histone mRNA levels seen after treatment of cells with inhibitors of protein synthesis alone could be consistent with autoregulation. If the rate of DNA synthesis is normally limiting the rate of chromatin biosynthesis, the same mechanism for the elevation of histone mRNA levels could operate in uninhibited cells as well as in those treated with inhibitors of DNA synthesis.

Another point sometimes overlooked in discussions about linkage between DNA synthesis and histone synthesis is that inhibition of protein synthesis also leads very quickly to inhibition of DNA synthesis (although a small amount of DNA synthesis can persist for a while after protein synthesis is inhibited).[423-425] These results suggest that DNA synthesis may be slowed as a result of the inhibition in the production of histone.

Other mechanisms have also been proposed to explain the linkage between histone and DNA synthesis. Stimac et al.[420] proposed that a labile protein may be involved in the coupling of the histone mRNA levels and the rate of DNA synthesis. When protein synthesis was inhibited, this labile protein would rapidly disappear rendering mechanisms regulating histone gene transcription and histone mRNA lability inoperative and uncoupling histone mRNA levels and DNA synthesis. However, this explanation by itself does not explain the decrease in histone mRNA levels when DNA synthesis is inhibited. It should also be noted that the concentration of free histone could reflect changes in the rate of protein synthesis just as the concentration of a labile protein could. The free histone would be incorporated into chromatin (with or without replication), instead of being degraded, as a labile protein would.

To explain their data, Graves and Marzluff[414] also suggested that a labile protein may

link the histone mRNA level and DNA synthesis, and further suggested that the lowering of histone mRNA levels in response to inhibitors of DNA synthesis may be mediated by changes in the nucleotide pools. The latter suggestion was based on the action of novobiocin, which inhibited DNA synthesis but did not alter either the deoxynucleotide pools or the histone mRNA level. However, novobiocin inhibits protein synthesis as well as DNA synthesis. The inhibition of protein synthesis would prevent any increase in the level of free histone when DNA synthesis was blocked. Thus the histone mRNA level would not be expected to fall when cells are treated with novobiocin. Helms et al.[417] and Baumbach et al.[416] have also suggested that either histones may regulate their own synthesis, or that synthesis of some other protein may be required for the coordination of DNA synthesis and histone mRNA levels.

Combining the ideas discussed above, a four-component model can provide a framework for explaining the changes in the level of histone mRNA after various treatments as follows:

1. The rate of chromatin replication can be limited either by the rate of DNA synthesis or by the rate of histone synthesis presumably mediated by the level of free histone.
2. The level of free histone also presumably regulates the level of S-phase histone mRNA through uncharacterized regulatory mechanisms.
3. Histones are synthesized and incorporated into chromatin at all times, very rapidly during replication and more slowly at other times; thus, the level of free histone could always be in a dynamic steady state, even when DNA synthesis is inhibited.
4. In uninhibited cells, the set point of the regulatory system seems to be with DNA synthesis limiting, so that the histone pool would be at an intermediate level and only a fraction of the potential maximum histone mRNA level would be present.

The histone pool mentioned in these models is a somewhat abstract term and the physical or biochemical nature of the pool is not known. Although measurements show that the histone mRNA levels for the various histone species seem to be regulated in unison,[413-418] this does not necessarily mean that one signal regulates all the levels. There is the related question of how the synthesis of the various protein species are maintained in approximately 1:1 ratios. Histones could exist in the pool as various oligomers or bound to some sort of pool or carrier protein. The pool has been estimated to be approximately 0.2% of total cellular histone[426] and there is some evidence that suggests that the levels of the arginine-rich histones, H3 and H4, may increase more than the levels of the moderately lysine-rich histones, H2A and H2B, when cells are treated with inhibitors of DNA synthesis.[427] However, because of the small size of the histone pool, it has not been studied in any systematic way. Thus, although the fragmentary results obtained to date are consistent with a model in which the level of free histone in the pool regulates the rate of histone synthesis, it is not known even if the level of free histone changes in a manner consistent with autoregulation.

Several groups have investigated the relative contributions of alterations in histone mRNA transcription and lability after treatment of cells with various inhibitors of DNA synthesis or protein synthesis. There is general agreement that when DNA synthesis is inhibited, histone mRNA transcription is decreased from two- to fivefold and histone mRNA lability is increased several-fold.[413,414,416,418] The half-lives of the histone mRNAs are about 1 hr in uninhibited mammalian cells in culture and decrease to values around 10 min after DNA synthesis is inhibited. Simultaneous inhibition of protein synthesis prevents these changes. When protein synthesis alone is inhibited, transcription rates increase somewhat (1.5- to 2-fold) but the mRNA lability decreases by at least a factor of four.[414,419] Thus, it seems that transcriptional rates can be altered approximately 7- to 8-fold during S phase and degradation rates by approximately 16-fold. As a rough estimate, these changes could together account for alterations in the histone mRNA levels of about 120-fold.

This discussion has been restricted to histone synthesized during chromatin replication. The relationship of histone and DNA synthesis at the beginning and end of S phase and during G1 are interesting and important questions. Inhibition of DNA synthesis leads to a shift in the pattern of synthesis of histone variants to the pattern found in G1 cells.[404] This suggests that the relationship of G1 to S phase when DNA synthesis is inhibited may be a close one. However, studies in vitro on transcription of various genes have suggested that the rates of transcription of histone genes are somewhat higher using soluble factors from S-phase cells vs. factors from G1 cells, while adenovirus genes are more efficiently transcribed using factors from G1 cells.[428]

Lower concentrations of cycloheximide which inhibit protein synthesis by 80% still permit slow exponential growth;[429] concentrations which inhibit protein synthesis by 40 to 50% have been reported to increase the length of G1 but not S phase.[430 430] These results have been interpreted in terms of a step in G1 that is particularly sensitive to the rate of protein synthesis. However, Wu and Bonner[431] have recently shown that the mechanism for this differential sensitivity is that the mRNA level for S-phase histones is specifically increased to offset the decrease in translation efficiency. Thus, within 2 hr after the initial inhibition, histone and DNA synthesis have recovered to their uninhibited rates, when protein synthesis is initially inhibited by less than 50%.

3. Histone Turnover

As mentioned previously, histones are synthesized and incorporated into chromatin not only in S phase but also in G1 and in the nonproliferation state G0. The rate of synthesis in G1 and G0 may be only a few percent of the rate during chromatin replication, but it is comparable to the rates of synthesis of many other proteins in G1 cells.

While G1 may last for only a few hours, G0 can presumably last for decades, as may be the case for mammalian neurons, which do not proliferate after birth. Thus, the effects of synthesis patterns of histone variants in nonproliferating cells should be manifested in the mass pattern of these cells.

In all nonproliferating mammalian cells examined, the only H3 variant synthesized was H3.3. These included contact-inhibited human embryonic fibroblasts (IMR-90), contact-inhibited, serum- or isoleucine-starved Chinese hamster ovary cells (CHO), confluent mouse 3T3 cells,[405] unstimulated human total lymphocytes as well as purified T-lymphocytes,[67] and a suspension-grown human leukemia cell line (HL60) induced with phorbol esters to stop proliferating and become attached.[432]

In unstimulated human lymphocytes, H3.3 dominated the mass pattern while the mass pattern of H3 in other cells grown to quiescence in tissue culture was still dominated by H3.1 and H3.2. When the lymphocytes were stimulated to proliferate, H3.1 and H3.2 synthesis began and after 72 hr those two variants dominated the mass pattern, indicating that lymphocytes could synthesize H3.1 and H3.2, and that the variant composition of lymphocyte chromatin could rapidly be altered.[67]

One reason nonproliferating tissue culture cells have not been found to shift their H3 mass pattern can be found in studies of the rate of histone turnover in organs of whole animals. Several studies, utilizing pulse-chase protocols with radioactive H_2O, thymidine, or amino acids in whole animals, have calculated half-life values of 159,[433]104, and 117 days[434] for histones in mouse brain, and 132 days for histones in rat brain.[435] Values of 117,[433] 93, and 105 days[434] have been found for histones in mouse liver. Clearly histone half-lives in nonproliferating cells can be quite long, thus it is no surprise that mass changes in H3 variants are not apparent in nonproliferating tissue culture cells after only a couple of weeks.

These experiments measuring histone turnover are not simple in execution or in interpretation. Histone turnover could be due to cell turnover, chromatin turnover in stable cells perhaps during DNA repair, or histone turnover on stable DNA. Originally, the first pos-

sibility was considered to be the appropriate explanation:[434] that histones lasted as long as the cell did; but careful simultaneous measurements showed that the histone half-lives were significantly shorter than the DNA half-lives. Commerford et al.,[433] using ^3H-H_2O to label mice for 9 months, found half-lives of 318 days for liver DNA vs. 117 days for liver histone, and 593 days for brain DNA vs. 159 days for brain histone.

The other possibility — DNA turnover in stable cells — has not been considered; the DNA turnover data have generally been taken as a measure of cell turnover. The rate of DNA turnover would be a reasonable maximum value for the rate of cell turnover, but there is no reason to exclude DNA turnover due to repair or random replacement in stable cells. The fate of histones during DNA repair is not known, but it is known that inducing DNA repair synthesis in quiescent cells does not lead to changes in either the rate or the pattern of histone synthesis.[67] Thus, DNA could be replaced without altering the histone variant composition, whether or not there was accompanying histone turnover.

If only certain variants are synthesized and incorporated into chromatin in nonproliferating cells, the mass variant pattern should change to reflect the altered synthesis pattern. Studies carried out by Zweidler[84] on mouse liver have clearly shown that the mass pattern changes gradually with the age of the mouse so that as the histones turn over, the S phase or replication variants (H2A.1, H2B.2, H3.1, and H3.2 in the mouse) are replaced by other variants. The H3.1 and H3.2 present in the livers of mice over 6 months old decay with a half-life of 360 days. This value is somewhat longer than the 117 days found by Commerford et al.[433] using pulse-chase protocols, but this would be expected since the low fraction of replicating cells contributes to the H3.1 and H3.2 mass measured by Zweidler,[84] but not to the radioactivity measured by Commerford et al.[433]

Table 3 shows the kinds of assumptions involved in making estimates of histone turnover. Assume initially that the mice have livers with 50% H3.1/H3.2 and 50% H3.3. The first process would be histone turnover on stable DNA (lines 3 to 5) at 0.41% per day. This histone would be replaced only by H3.3 since the cells are not going through an S phase. If this were the only process involved, the amount H3.1/H3.2 would be expected to decrease with a half-life of 185 days. Working in the opposite direction is cell turnover which tends to increase the amount of H3.1/H3.2 (lines 6 to 8). In this calculation, cell death is assumed to be independent of the time since its last cell division, and the rate of 0.24% per day is from Commerford et al.,[433] who measured DNA turnover. These cells are replaced by replication; the histones could have an S-phase pattern H3.1 + H3.2/H3.3 of 10 as found in the livers of young mice. The net estimated decrease is 0.105/50 or 0.21% per day for a $t_{1/2}$ of 362 days. The rate of decrease of H3.1 + 3.2 found by Zweidler[84] was 0.2% per day for a $t_{1/2}$ of 360 days.

The agreement between different methods is reassuring, but there are still questions. There seems to be a significant difference in cell turnover as estimated by the decrease in radioactive DNA and by the percent-labeled nuclei. Zweidler[84] reported values of about 0.1% per day by the second method compared to 0.24% per day reported by Commerford et al.,[433] using the first method. Commerford et al.[433] suggest that autoradiographic results might be artificially low, but do not suggest how. One possibility is that there is a slow DNA repair-type synthesis in these cells of the order of (0.24 to 0.10%) 0.14% per day ($t_{1/2}$ = 540 days). This could reconcile the differences between these two types of results. DNA turnover in stable cells would not affect the calculation of histone turnover (lines 3 to 5) since neither the variant pattern nor the amount of histone synthesis is changed when DNA repair synthesis is induced.[67]

If the value of 0.1% per day is a more accurate reflection of cell turnover, the calculation yields an apparent half-life of 232 days for H3.1 + H3.2. If cell death is not assumed to be random, but only the oldest cells (with only H3.3) die, then an apparent half-life of 338 days is calculated for H3.1 + H3.2.

Table 3
ESTIMATES OF HISTONE TURNOVER[a]

Variant	H3.3	H3.1/ H3.2	
Amount t = 0 day	50.000	50.000	
Histone turnover			
Random removal (0.41%/day)	−0.205	−0.205	$t_{1/2}$ = 185 days
Replacement by H3.3	+0.410	—	
Net histone turnover	+0.205	−0.205	
Cell turnover			
Cell death (random, 0.24%/day)	−0.12	−0.12	
Cell replacement (S phase)	+0.02	+0.22	
Net cell replacement	−0.10	+0.10	
Net change	+0.105	−0.105	$t_{1/2}$ = 362 days
Amount t = 1 day	50.105	49.895	

[a] See text for details and explanation of the calculations.

Some studies of histone turnover in mammalian liver and kidney have resulted in much shorter half-lives of 4 to 15 days for mouse kidney histones vs. 20 days for the kidney DNA, and similar values for the liver.[436] The explanation for these values may be in the heterogeneous cell population of these organs. Liver has two cell populations with distinctly different turnover times. Commerford et al.[433] found that 23% of mouse liver DNA had a half-life of 12 days and the other 77% had a half-life of 318 days. Thus, the short term experiments only measured the faster process. The short half-lives found in the short-term radioactive pulse-chase experiments are not reflected in the half-lives of variants as measured by mass.[84] This result confirms the importance of knowing the life cycles of all the cells in the tissue under study. Brain does not contain such a rapidly turning over DNA component, thus accounting for the better agreement of values in the literature for histone turnover in brain. Grove and Zweidler[437] have also found shorter half-lives for some histones in differentiating MEL cells in culture.

The mechanisms of histone degradation during turnover is not known. Two possibilities could be mentioned. Histones could be degraded directly from chromatin, then replaced by histones from the pool. Alternatively, histones in the pool could be in some sort of equilibrium with histones in chromatin. Then degradation could take place directly from the histone pool. The evidence that does exist supports the former rather than the latter; in short pulses of radioactive amino acids, most of the radioactivity enters chromatin rapidly. However, careful studies to distinguish these two possibilities have not been done.

B. Nucleosome Assembly

In the previous sections we have reviewed the synthesis of the protein component of chromatin and the relationship between histone and DNA synthesis. In this section we are concerned with the packaging of the newly synthesized histones and their deposition onto chromatin as well as segregation of the "old" core particles at the replication fork. Only nucleosome assembly during replication will be discussed. Much conflicting data exist in this area of research. A major problem is that the experimental approaches and techniques used by different groups are sufficiently different so that it is not readily apparent how to resolve the disagreements. Furthermore, there is no standard model system where all the different techniques can be tested and compared.

Studies of nucleosome assembly fall into two areas. The first area is concerned with the relationship of newly synthesized (new) histone protein with preexisting (old) histone protein

within an octamer. The second area is concerned with the relationship of the histone proteins with the two strands of DNA being generated at the replication fork.

In the first area, Leffak et al.[438] proposed three possibilities for the assembly of newly made histones. These are (1) random assembly — new and old histones are randomly incorporated into octamers; (2) semiconservative assembly — old tetramers (H2A, H2B, H3, H4) pair with new tetramers; and (3) conservative assembly — new histones do not mix with old histones and form octamers of all new histones. Jackson and Chalkley[439] proposed another possibility — new H2A-H2B dimers pair with old H3-H4 dimers and the hybrid tetramers are then assembled into octamers leading to a random pattern of octamer assembly.

In the second area, two models for segregation of parental histones to daughter chromatids at the replication fork are being actively discussed.[248,440-442] They are (1) random distribution — old histone cores are distributed to daughter DNA in a random manner and (2) nonrandom distribution — all old histone cores distributed to one of the daughter strands. These two models have meaning only if octamers are assembled conservatively, that is they can be traced as old or new.

The experimental approaches taken to differentiate these possibilities have been very divergent. Leffak et al.[438,443,444] have labeled nascent histones by growing cells in a medium which contained dense amino acids plus ^{3}H-lysine. These cells were mixed with excess unlabeled cells and nucleosomes prepared from the isolated nuclei by staphylococcal nuclease digestion. The soluble chromatin fraction (25 to 30% of the total chromatin) was depleted of H1 and a portion of the H1-depleted chromatin was cross-linked with the reversible cross-linker dithiobissuccinimidyl propionate. The cross-linked chromatin was analyzed on buoyant density gradients under denaturing conditions. Under these conditions, the cross-linked histone core particles separate from the DNA and are distributed in the gradient according to their content of dense amino acids. After reversing the cross-links, the different fractions from the density gradient were further analyzed on SDS PAGE for their histone content. Due to the density distribution of the radiolabeled histones, Leffak concluded that the nascent histones were deposited and segregated as intact octamers. Thus, the "old" and nascent histones do not mix and the process of assembly occurs in a nonrandom conservative manner. In another series of experiments, Leffak[443] showed that even after five to six cell generations the original dense octamers remain intact as a dense unit.

Analysis of the cross-linked dinucleosome band showed that immediately after the pulse period the dense octamers are adjacent to each other; after five to six rounds of replication (cell generations), the dense octamers are now adjacent to normal density octamers. Two interpretations of the data were presented. In the first, the octamers were segregating randomly with respect to the DNA strands during each cell generation. In the second, if a domain of oligonucleosomes segregated conservatively with respect to the DNA strands, then the boundaries of such a domain varied during each cell generation. Assaying the shift of cross-linked dinucleosome density from heavy-heavy to heavy-light with cell generation might differentiate these two models.

Jackson and Chalkley,[439] using a different cross-linking procedure, obtained results different from those of Leffak et al.[438] Their method took advantage of the nuclease digestion properties of nascent chromatin and the ability of CsCl-GuCl density gradients to resolve nascent chromatin from bulk chromatin. Formaldehyde was used to fix the cells thus freezing all the proteins associated with DNA at any particular time via protein-DNA cross-links. The fixed chromatin was solubilized by mechanically shearing in 2 *M* NaCl-5 *M* urea and then analyzed on CsCl-GuCl gradients. The fractions of the CsCl-GuCl gradient were further analyzed for their histone content on SDS PAGE after reversing the DNA-protein cross-links with mercaptoethanol. They found that the newly synthesized H3 and H4 were deposited onto new DNA and remained with the "new" DNA during a long chase. The nascent H2A-H2B however were bound transiently to new DNA and were largely associated with preex-

isting chromatin. If H3 and H4 are deposited onto DNA differently from H2A and H2B, then this result is clearly inconsistent with the concept of conservative assembly of all four new core histones into a single unit.

Russev and Hancock[445] studied the assembly of new histones into nucleosomes and their distribution in replicating chromatin by density labeling the protein and DNA, nuclease digesting the chromatin, and separating the nucleosomes on metrizamide gradients without prior cross-linking. They determined that 74% of new histones become complexed with the new DNA and about 26% with nonreplicating DNA. Furthermore, the majority of new histones formed entirely new nucleosomes with a minor fraction forming hybrid nucleosomes that also contained preexisting histones. The new nucleosomes were distributed to both new daughter DNA molecules with approximately equal probability. Since new histones are not deposited exclusively with new DNA, then nucleosomes or histones from other regions of chromatin must migrate to give a random pattern of segregation. Recent studies by Fowler et al.[446] confirm a random distribution of histone cores and the ability of old octamers to migrate to new positions.

Seale and co-workers[447-449] studied the assembly of newly synthesized histones and have reported results that support the model of Jackson and Chalkley.[439] They found that H2A and H2B can enter the bulk chromatin fraction, whereas H3 and H4 are first associated with a nascent chromatin fraction and with a lag appearing in bulk chromatin. Their conclusions were based on differential salt extractability of nascent H2A-H2B vs. nascent H3-H4 from chromatin isolated from cells that were pulse labeled with ^{3}H-lysine for 2 min. All the nascent H2A and 70% of nascent H2B were salt stable and remained with polynucleosomes, whereas about 30% of nascent H2B, 50% of H4, and all of H3 were salt labile. Thus, the assembly of the core octamer appeared to be random and nonstoichiometric. Annunziato and Seale,[248,449] in another series of experiments, examined the nuclease sensitivity and the unit repeat size of bulk chromatin and nascent chromatin. They concluded that segregation of nucleosomes at the replication origin is random. That is, "old" octamers are distributed to both arms of the replication fork.

Seidman et al.[441] used another approach to study nucleosome segregation. They asked whether the nucleosome segregation process on SV40 chromosomes in cycloheximide-treated cells was random/dispersive (nonconservative) or nonrandom (conservative). Their approach took advantage of the SV40 chromosome which in addition to having a typical complement of histone octamers has a specific origin of replication. Thus, one can differentiate the two arms of the replication fork by DNA hybridization and assess the degree of segregation of histone octamers onto the two daughter strands by nuclease digestion. They found that the nuclease-resistant SV40 DNA daughter strands, newly replicated in the absence of protein synthesis, hybridized predominantly to the template strand on the leading side of the replication fork. Thus the result is consistent with the idea that the parental histones preferentially segregated to and protected the leading and not the lagging DNA strand from nuclease digestion. This conclusion supports a nonrandom (conservative) mode of nucleosome segregation.

Recently, Cusick et al.[442] repeated this same series of experiments and reached the opposite conclusion that the nucleosomes segregated in a random/dispersive (nonconservative) manner. This conclusion was reached because they found the nascent DNA annealed equally well to both strands of SV40 DNA. This disaccord may be explained by the way the hybridization process was performed. Seidman et al.[441] may have lost some of the DNA sequences blotted onto nitrocellulose membranes during the hybridization procedure. With the advent of improved technology such as DBM-paper and Gene Screen®, Cusick et al.[442] did not have the same technical problems encountered by Seidman et al.[441] and managed to recover all the relevent sequences blotted onto DBM-paper or Gene Screen®.

The key to resolving the conflicting models of chromatin replication and nucleosome assembly is to determine if and how histone octamers are assembled prior to incorporation

into nucleosomes. If histone octamers are not assembled as indefinitely stable units or if they migrate on the DNA strands, then the arguments concerning the manner of segregation of "old" octamers to daughter DNA strands become moot. This is because many of the models operated on a basic assumption that the nucleosomal component of newly replicated DNA derives from segregation of parental prefork octamers to newly replicated daughter double helixes with little change in position. Thus many of the experiments may have been improperly designed. Additional studies including the steps between the release of histone polypeptide chains from polyribosomes and their integration into nucleosomes would be useful in reaching a concensus. There is evidence that a small free histone pool exists in the cytoplasm of cells. By studying the variant composition of the pool, the core histone ratio of the pool, and any exchange with histones in the nuclear compartment, the mechanism of chromatin assembly may be further elucidated.

C. Nucleosome Positioning

During the process of replication, are the nucleosomes positioned in a definite way relative to particular DNA sequences, nonhistone proteins, or other factors? This question has become more relevant recently as evidence accumulates that hypersensitive regions in active genes may be free of nucleosomes (see Section VI). Arguments both for and against nucleosome positioning or phasing have been presented (reviewed in References 450 to 453). As discussed in previous reviews, this has been a controversial topic because most methods to align nucleosomes on a given DNA sequence have relied on the ability of endonucleases to gain preferential access to the DNA in chromatin which is not protected by the nucleosome core. None of the enzymes utilized can provide this function in the most useful way. Micrococcal nuclease has been used most often because it more readily digests the DNA which is not directly bound to the nucleosome core particle. The very important drawback to the use of this enzyme is the fact that it exhibits a certain degree of sequence specificity in its cutting of DNA, in particular, (A + T) rich sequences have been found to be preferentially cleaved.[454-456] Careful comparison of the cleavage of naked DNA and chromatin must be done. DNase I has been utilized in many studies of the chromatin of actively transcribed genes (see Section VI). This enzyme, however, is not as useful for the mapping of the position of nucleosomes along the DNA sequence because it makes single strand breaks in the core DNA and spacer DNA simultaneously.[177,457] Several recent studies appear to have obviated these difficulties in determining nucleosome positioning or phasing.

Gottschling and Cech[458] have conducted a study of the chromatin structure at the telomeric ends of *Oxytricha* macronuclear DNA. The macronucleus of this single-celled eucaryotic contains approximately 24 million gene-sized DNA molecules, each having terminal C_4A_4 repeat sequences. Using a radioactive probe to the terminal repeats as an indirect end-label, they were able to show that phasing extended up to 6 nucleosomes in from a protected 100 base-pair long terminal complex. Their interpretation seems quite accurate for several reasons. Two independent methods of probing the DNA-protein interactions were used, micrococcal nuclease and a new DNA cleavage reagent with low sequence specificity, methidium propyl-EDTA·Fe(II) or MPE·Fe(II).[459] In this particular study, the known sequence specificity of micrococcal nuclease is likely to be irrelevant since a large and heterogeneous population of DNA sequences, rather than those of individual genes, are being probed. Additionally, as in most careful studies now, the cleavage patterns in chromatin were compared to those in naked DNA.

Using radioactively labeled probes from either the *Oxytricha* ribosomal RNA genes or the C2 gene, they also tested for the presence or absence of nucleosome phasing in from the telomeric ends of particular macronuclear DNA molecules. The phasing of nucleosomes in from the telomeric ends was more clearly evident from one end than from the other in the 8.2-kilobase long DNA molecule containing the ribosomal genes. The *Oxytricha* C2

DNA molecule, on the other hand, is only 0.810 kilobases long; therefore, it is not so surprising that they observed three nucleosomes "phased" between the 100 base-pair terminal complexes. A more conclusive case could have been made for the phasing of nucleosomes on each of these DNA molecules if additional experiments had been done using MPE·Fe(II) in place of micrococcal nuclease.

Edwards and Firtel[460] have studied the chromatin structure of the 10-kilobase spacer region which lies between the 3′ end of the 36S rRNA coding region and the telomeric end of the *Dictyostelium* rDNA molecule, which is a linear, extrachromosomal, palindromic dimer of about 88 × 10^3 bases in length. The telomeres are analogous to those of *Tetrahymena*, *Physarum*, and *Oxytricha*, consisting of a simple repeat, $(C_nT)_m$, of 100 to 200 base pairs in length. They were able to demonstrate a specific phasing of nucleosomes along the 10 kilobase pairs of DNA by using the indirect end-labeling technique to map nucleosome positions from both ends of this region. Notably, they observed that those sites which were most nuclease susceptible in naked DNA were highly protected from cleavage in chromatin. The phasing was found to be present in nuclei which had been washed with 0.55 *M* KCl, but not after the nuclei were washed with 0.75 *M* KCl, demonstrating that nucleosome cores were responsible for the cleavage patterns observed. In 0.75 *M* KCl, the cleavage patterns were typical of that seen with naked DNA, suggesting that the nucleosome core had been randomized or altered at this KCl concentration.

The α-satellite DNA of the African green monkey is a 172 base-pair repeat sequence which comprises approximately 15 to 20% of the green monkey genome.[461] The chromatin of this satellite DNA has been actively studied with particular attention given to determining whether or not there is a particular register of nucleosomes along the repeated sequence [462,463] (reviewed in References 450 to 452). Strauss and Varshavsky[464] have taken a different approach to this question. Using a gel electrophoresis band competition assay which allowed the detection of specific DNA-binding proteins in crude extracts, they found a protein in extracts from purified nuclei of green monkey CV-1 cells that was able to form a specific complex with the 172 base-pair α-DNA monomer. The specific α-DNA-binding factor (α-protein) was purified to homogeneity and was found to be similar in some ways to HMG 14 and HMG 17. Utilizing the technique of DNase I footprinting, the authors found that the α-protein bound to three specific sites in the 172 base-pair α-DNA sequence. Two of the binding sites (sites II and III) were spaced 145 base pairs apart and were located at positions which had been previously determined[465] to be at the entry and exit positions of the DNA from the nucleosomes of EcoR1 solubilized α-satellite chromatin. Moreover, these binding sites were determined to be inverted seven base-pair repeats of each other (5′ -AAATATC and 5′-GATATTT). The third site (site I) was located halfway between these two sites and involved a weakly homologous seven base-pair sequence (5′-TTAATTC). If the nucleosome core is actually phased in this way on the α-DNA-repeat sequence, then all three sites would be near each other in the region of the nucleosome core where the DNA enters and exits. As a final comment, it must be noted that such a nucleosome phasing for the bulk of α-satellite chromatin has recently been questioned by Smith and Lieberman,[462] who have found that there are probably a minimum of three different phase relationships of nucleosomes along α-satellite DNA.

Many nonhistone proteins with various DNA-binding characteristics are being studied.[466-474] The binding of α-protein to this region underlines the similarity of this protein to HMGs 14 and 17 since these proteins have previously been shown to bind to this region of the nucleosome.[469,470]

Thoma et al.[475] have studied the chromatin structure of an extra-chromosomal circular yeast DNA (TRP1ARS1) of 1453 base pairs in length. This DNA contains the *N*-5′ phosphoribosyl anthranilate isomerase (TRP1, map units 103 to 775) gene and a sequence which functions in autonomous replication (ARS1, map units approximately 775 to 1453). Specific

restriction fragments were obtained from these DNA sequences and utilized for indirect end-labeling experiments in which micrococcal nuclease and DNase I cleavage sites were mapped. They observed sequence-specific cutting of naked DNA, but found that the intensity of bands was quite different in chromatin than in naked DNA. Their analyses indicated that three nucleosomes were stably positioned on the ARS1 region. However, the chromatin structure of TRP1 region seems to be more complicated. A nucleosome repeat pattern of monomers, dimers, and trimers was observed when micrococcal nuclease fragments were probed with a labeled restriction fragment from the TRP1 region, but the few sharp bands observed at low levels of nuclease cleavage quickly disappeared with increasing digestion. They concluded that either the four nucleosomes of the TRP1 region are unstable and not resistant to nuclease cleavage or they are organized in several different phase arrangements, each with a different nuclease susceptibility. The two regions within the circular DNA which separate the ends of TRP1 and ARS1 (region A, map units 676 to 862; and region B, map units 1341 to 66) were especially sensitive to nuclease cleavage. The degree of nuclease sensitivity indicates that the DNA of these regions is not organized into nucleosomal structures, but the cleavage pattern does suggest that other protein:DNA interactions might be present. Since it is possible that these nucleosome-free regions may form boundaries from which nucleosomes are phased it was not possible from the experiment to determine the actual contribution of the underlying DNA sequence of nucleosome positioning.

Bloom and Carbon[476] have examined in detail the chromatin structure of the centromere regions of chromosomes III and XI in yeast through use of cloned functional centromere DNA (CEN3 and CEN11) as labeled hybridization probes. The DNA fragments resulting from micrococcal nuclease digestion of chromatin in isolated yeast nuclei were electrophoretically resolved into a ladder of nucleosomes with repeat sizes more distinct than that observed for the bulk of the chromatin or even for that found by hybridization probing of another gene region which is located 20 kilobases downstream from the centromeric DNA sequences. Control digestions of deproteinized chromatin failed to produce any distinct pattern of the centromeric DNA fragments. The largest centromeric fragments were found to correspond to 15 or 16 nucleosomal subunits, each containing 160 base pairs of DNA. They interpreted the sharp resolution of these centromeric DNA fragments to be due to a greater uniformity of the internucleosomal spacers in centromeric chromatin as compared to bulk chromatin. When the positions of the micrococcal nuclease and DNase I sensitive sites in the centromeric chromatin were mapped by indirect end-labeling technique, a distinct 250 base-pair region of the CEN sequence was found to be protected from nucleolytic cleavage. This region was, however, found to be bordered by micrococcal nuclease and hypersensitive DNAse I cleavage sites. This method of position mapping showed the nucleosome subunits to be ordered and specifically aligned on the yeast DNA which flanked the 250 base-pair protected region of centromeric DNA. Extraction of the chromatin with a salt concentration high enough to disrupt nucleosome core structure led to the production of the same sequence-specific nuclease cleavage patterns seen with naked DNA.

The authors also found that the structural organization of chromosomal CEN sequences just described was maintained when this region of the yeast genome was cloned into autonomously replicating plasmids and analyzed as above. In an elegant series of experiments, the authors demonstrated that the yeast sequences which flank the 250 base-pair CEN DNA are required for the characteristic nucleosomal array found in this region of the chromosomes. They constructed a series of autonomously replicating plasmids with which the relative contributions of the different centromeric DNA sequences to the described chromatin structure could be tested. The nucleosome positioning was not altered if only the length of the yeast DNA flanking the CEN regions was varied, or even if the CEN-sequence DNA was deleted. However, substitution of the yeast DNA sequences which flank the CEN3 region with either pBR322 DNA or other yeast DNA sequences eliminated this ordered nucleosome

positioning. They concluded that the nucleosomal phasing described for the 3.5 kilobases of DNA in the CEN region is dependent on either the base composition or actual DNA sequence and not simply as the result of a boundary created by the exclusion of nucleosome cores from the 250 base-pair nuclease-resistant CEN site.

Simpson and Stafford[477] reconstituted chicken core histones onto a defined 260 base-pair fragment of *Lytechinus variegatus* DNA which contains a 5S rRNA gene. A certain proportion of the DNA in the reconstitutes was end-labeled allowing them to map with some precision the sites of DNAse I cutting on both strands. A "footprint" of more nuclease-resistant DNA was detected from about nucleotide position 21 to position 165 of the 260 nucleotide-long fragment (the 5S rRNA gene is located from position 91 to 210). The authors concluded that the information inherent in the DNA sequence (or base composition) and in the histone complexes is sufficient to mediate a site-specific positioning of a nucleosome. Although the chromatin of sea urchin nuclei has not yet been examined in this regard, four different nucleosome phase relationships have been described for the *Xenopus* 5S rRNA gene[478] and two different nucleosome phase relationships have been described for the *Drosophila* 5S rRNA gene.[479] One of the phase arrangements found in the *Drosophila* 5S gene corresponded to the specific nucleosome positioning reconstituted in vitro on the 260 base-pair fragment of DNA containing the *Lytechinus* 5S rDNA. A more complete discussion of 5S gene chromatin and its transcription is presented in Sections VI.B and C.

These studies have clearly shown that at least in some regions of the genome, nucleosomes are not randomly located. Whether the nonrandomness is a secondary result of other events or is an essential element of chromatin structure and function remains to be discovered.

VI. HISTONES AND TRANSCRIPTION

Many studies have suggested that nucleosomes are present in transcribed as well as nontranscribed regions of the chromosome (reviewed in References 450 to 452 and 480). The first suggestion of this possibility came from studies in which it was found that nuclease-resistant chromatin subunit structures contained the DNA sequences necessary to encode the RNA purified from the same cells.[481,482] Many electron microscopic studies have been conducted to define the structural differences between chromatin of active and inactive gene regions. In a number of these studies nucleosomal structures were not observed in the active transcriptional units of the chromatin containing the genes for ribosomal RNA.[483-491] Others found that active transcriptional units in nonribosomal RNA-encoding chromatin contained a repeating nucleosomal structure.[486] Proper characterization of the morphology of transcribing chromatin by electron microscopy is at best difficult due to the presence of the RNA polymerase and other possible accessory factors as well as the unnatural conditions of sample preparation. Biochemical analyses have indicated that histones are present on the active chromatin regions of the ribosomal genes.[492-495] The presence of histones on transcribed DNA sequences was demonstrated by immunoelectron microscopy.[496] It is important to note that histones can become nonspecifically bound to transcribed DNA sequences of the chromatin during sample preparation for the electron microscope.[497]

Most methods which have been used to distinguish transcriptionally active from inactive gene regions in the chromatin are based on the differential accessibility of these regions to nucleases. Weintraub and Groudine[498] were the first to demonstrate that the active gene sequences within intact nuclei were preferentially digested by DNase I. Their results were confirmed by analysis of other active gene sequences in a variety of organisms and tissues[499-501] (reviewed in References 253 and 451). The differential sensitivity to DNase I does not necessarily provide a probe for on-going transcriptional activity as the alterations in chromatin structure which confer this sensitivity are often maintained after the transcriptional event [502,503] (reviewed in References 451 and 452). In contrast, for the *Drosophila*

heat shock genes, genetic activity was paralleled by an increased DNase I sensitivity and this sensitivity was lost upon the cessation of transcription of these genes.[504,505] Wu[506] refined the analysis of the DNase I sensitivity of the heat shock genes of *Drosophila* embryos and tissue culture cells with his observation of distinct sites to the 5′ side of these genes which were hypersensitive to the action of this nuclease. This phenomenon has since been shown to be common to the activation for transcription of a wide variety of genes and typically involves short regions of about 50 to 400 base pairs in length to the 5′ side of the gene (reviewed in References 451, 452, and 507). DNase I hypersensitive sites have also been found to the 3′ side of some genes.[508,509] The chromatin of genes which are not actively expressed but have the potential for expression have often been found to be maintained in a nuclease hypersensitive state irrespective of the level of transcriptional activity.[510-512] It has also been found that specific patterns of hypersensitive sites can be a reflection of the state of transcription of particular genes.[513] Regions of chromatin which exhibit moderate degrees of sensitivity to DNase I have been identified in sequences 20 to 50 kilobase pairs upstream of the transcribed genes.[514,515]

The sites of DNase I hypersensitivity in the chromatin upstream from active or potentially active genes have been the subject of careful experimental scrutiny (reviewed in References 451 and 452). It has been suggested that these sites may be situated in short nucleosome-free regions of the chromatin which allow the regulatory proteins to bind to their particular target DNA sequence.[516] Wu[517] developed an *in situ* Exo III protection technique that permitted mapping of the DNA sequences within the nuclease hypersensitive sites in chromatin onto which the regulatory proteins might bind. In several *Drosophila* heat shock genes, the resistant regions were found to cover the TATA box sequence and an upstream control element. Several studies are beginning to provide insights into the roles of histones in transcription.

McGhee and co-workers[516] identified a nuclease hypersensitive region which spanned the region from about 60 to 260 base pairs upstream from the 5′ start site of transcription of the chicken adult β-globin gene and which was directly related to the transcriptional activity of this gene. They found that a 115 base-pair DNA fragment could be excised from this region by digestion with the restriction enzyme, Msp I, with a greater than 50% yield, suggesting that this hypersensitive region could quite possibly be free of histones. Emerson and Felsenfeld[518] utilized this system to search for factors which might bind to these upstream control sequences of the β-globin gene. Plasmid DNA which carried the β-globin genes was combined in vitro with histones in the presence of extracts from 9-day-old chicken erythrocyte nuclei (which contain active β-globin genes in vivo). The upstream sequence in the nucleoprotein complex formed on the plasmid DNA was hypersensitive to nuclease digestion. If, however, the nuclear extracts used during the assembly of histones onto the plasmid DNA were obtained from cells not expressing the β-globin gene, the typical upstream hypersensitive region was not observed.

Their second method of assay for reconstitution of the upstream hypersensitive site took advantage of the known ability of Msp I to release a 115 base-pair fragment from this site. In this assay, the artificial nucleoprotein complexes were assembled in the presence of various nuclear extracts, then treated with Msp I. They found no selective release of the Msp I fragment when the active chicken nuclear extract was added after the histones were assembled on the DNA substrate. If, however, the active extract was added either before or together with the assembly factors and histones, subsequent digestion with Msp I led to preferential excision of the 115 base-pair fragment from the 5′ hypersensitive region of the β-globin gene.

When partially purified nuclear extracts were mixed with end-labeled restriction fragments of the β-globin gene, all were retained on a nitrocellulose filter with the same efficiency. When *E. coli* DNA was added to the mixture to compete for proteins that bound nonspe-

cifically, the filter preferentially retained the fragment which contained the 5′-flanking region of β-globin gene. The hypersensitivity to DNase I, DNase II, and Msp I digestion of the upstream region of the β-globin gene was only observed if this gene was reconstituted into a nucleoprotein structure in the presence of nuclear extracts from cells which were expressing the β-globin gene. In addition, the particular hypersensitivity of this region is not obtained when the factor is added after histone assembly, suggesting that it must recognize specific DNA sequences. Evidently, these sequences were masked and unavailable for factor binding when the histones were already bound to the DNA.

Recently, Larsen and Weintraub[519] found that the DNase I hypersensitive regions to the 5′ side of the chicken α- and β-globin genes in erythrocyte nuclei were sensitive to digestion by S1 nuclease. Since this nuclease is specific for single-stranded DNA, it was likely that the sensitive sites contained an altered DNA structure. They found that these sites were also sensitive to S1 nuclease when present in supercoiled plasmid DNA, but not if they were present in a relaxed circular plasmid DNA. Weintraub[520] found that the sensitivity of these sites, although reduced, was retained when histones were reconstituted to supercoiled plasmid DNA molecules containing such sites. He concluded that modification of DNA secondary structure in these regions caused a reduction in the ability of the core histones to bind and form a nucleosomal structure. McKnight and Kingsbury[521] independently arrived at a similar conclusion from their analysis of the in vitro mutagenesis of the transcriptional control sequences of the herpes virus thymidine kinase gene.

These studies on the β-globin gene indicate that the generation of hypersensitive sites to the 5′ side of this gene may involve mechanisms which prevent nucleosome formation at regions in the DNA sequence that are either maintained in a specialized secondary structure or are bound by specific transcriptional regulatory factors. Emerson and Felsenfeld[518] concluded that there was no contradiction between these two mechanisms.

Bergman and Kramer[522] analyzed the chromatin structure of a phosphate-repressible acid phosphatase gene (PH05) in the nuclei of the yeast, *Saccharomyces cerevisiae*. The authors found that the DNA of this gene region was more nuclease-sensitive in nuclei from cells grown in conditions which derepress the activity of this gene (low phosphate media) than when grown in conditions which depress its activity (high phosphate media). The DNA of this gene was found to be packaged into regularly spaced nucleosomes in both of these growth conditions, but a unique phase relationship of the nucleosomes to the DNA sequence of this gene was only observed when the activity of this gene was repressed during cell growth in high phosphate media.

Mirzabekov and co-workers[523] have applied their previously developed cross-linking and DNA cleavage techniques for the mapping of specific histones along the nucleosomal core DNA[225,226] to the analysis of chromatin structure in transcribed and nontranscribed regions of the genome. A portion of the DNA fragments produced by the cross-linking and DNA cleavage treatments of isolated *Drosophila* nuclei was separated in a two-dimensional gel electrophoretic system into three diagonals which correspond to free DNA, DNA cross-linked to core histones, and DNA cross-linked to histone H1. The proteins cross-linked to the DNA were removed *in situ* by protease treatment and the DNAs were transferred to DBM-paper prior to hybridization with probes from different regions of the hsp 70 gene. In accord with the known DNase I hypersensitivity of the 5′ region of this gene,[504-506] probes to this region only hybridized to the DNA diagonal, thereby indicating a lack of bound histones. Probes to the coding sequence of this gene hybridized to all three diagonals when the DNA fragments were isolated from the nuclei of *Drosophila* cells grown in nonheat shock conditions. Progressive depletion of the hybridization of coding region sequences in the diagonal of cross-linked histone H1-DNA and then in the diagonal of cross-linked core histone-DNA occurred with the increasing levels of transcription of this gene. This occurs when the temperature for cell growth is raised from 25°C to heat shock conditions (37°C).

Similar observations were made when the 3′-flanking region was probed in these same situations. This may be due to continuation of transcription past the 3′ end of the mature mRNA coding sequence. Caution must be taken not to interpret these results as proof of complete histone displacement from an actively transcribed gene. First, only about 10% of the histones are covalently bound to the DNA of isolated nuclei via their lysine ϵ-amino groups and *N*-terminal amino groups. The DNA fragments, including those which were cross-linked to protein, were concentrated by precipitation as a cetavalon salt and then this preparation was enriched in cross-linked protein-DNA complexes by adsorption to hydroxylapatite. The investigators are therefore studying only a fraction of the chromatin regions in question. Second, since this technique involves the cross-linking of chemical groups (lysine ϵ-amino and *N*-terminal amino groups) in the histones which are preferentially localized in the unstructured amino- and carboxyl-terminal domains of these proteins, the failure to detect the cross-linking of histones to transcribed sequences could potentially be the result of the transient displacement of these domains from the DNA, while the hydrophobic core of the histone subunits remains bound to the DNA (see Section III).

The *Xenopus* 55 RNA genes have provided one of the better model systems for the analysis of the transcriptional control of eucaryotic genes. In *Xenopus*[524] there are two types of 5S RNA genes which are differently regulated. The somatic-type gene is present at approximately 400 copies per haploid genome while the oocyte-type gene is present at approximately 20,000 copies per haploid genome.[524,525] The oocyte-type 5S RNA genes encode the bulk of the 5S RNA present in growing oocytes, but are not expressed in somatic cells. The somatic-type 5S RNA genes encode only a small portion of the 5S RNA produced during oogenesis, but account for virtually all of the 5S RNA that is synthesized in somatic cells.[526,527] These genes were found to be transcribed accurately when purified chromatin was used as a template for in vitro transcription.[528,529] Accurate transcription of the cloned forms of these genes could be demonstrated if the cloned DNA was preincubated in a *Xenopus laevis* nuclear extract.[530] Three factors in addition to RNA polymerase III were found to be necessary for the transcription of both of these cloned 5S RNA genes. Analysis of deletion mutants showed that the proper transcription of these cloned genes is dependent upon an intact intragenic control region which spans nucleotides +50 to +83 of the 120 base long gene.[531,532] One of the three transcription factors (TF IIIA) was found to bind to this region and act as a positive regulator of transcription.[533,534] Sakonju and Brown[534] observed that this factor bound four times more tightly to this internal control region of the somatic gene as compared to the oocyte gene.

Using a transcription assay, Bogenhagen et al.[529] found that when somatic cell chromatin was used as a template in an in vitro transcription assay the somatic 5S RNA genes were expressed but the oocyte 5S RNA genes were not. If this chromatin were washed with 0.6 *M* NaCl prior to the transcription assay, both genes were active.

In a subsequent study, Schlissel and Brown[535] prepared somatic cell chromatin and derepressed the oocyte gene by either treating the chromatin with 0.6 *M* NaCl followed by column chromatography to remove the extracted proteins or treating it with the cation exchange resin BioRex-70® in low ionic strength conditions. Both treatments have been used to remove histone H1 and many nonhistone proteins from chromatin.[536] These treatments did not inactivate the expressed somatic gene or activate the repressed oocyte gene. If, however, all of the transcription factors including RNA polymerase III were incubated with this chromatin, the oocyte gene was now also transcribed. The oocyte gene chromatin could be repressed if the removed H1 or an appropriate amount of exogenous H1 were added back to this chromatin. Core histone complexes [(H2A-H2B) or $(H3\text{-}H4)_2$] could also re-repress the oocyte genes but higher relative concentrations were necessary. These histone additions did not affect the active transcriptional state of the somatic 5S RNA genes. In a related observation, they found that the oocyte 5S RNA genes of different preparations of erythrocyte

chromatin were repressed to varying degrees. The level of repression appeared to be related to the amount of intact histone H1 remaining. These findings led them to conclude that histone H1 binding is probably involved in the repression of the oocyte 5S RNA gene by preventing the binding of the proper transcriptional factors to the intragenic control region of the oocyte gene in somatic chromatin. In somatic cells, the active transcriptional factors gain preferential access to the intragenic control region of the somatic 5S RNA genes. Once formed, these active transcriptional complexes are stable in vitro to both histone H1 removal and reconstitution.

The ciliated protozoan, *Tetrahymena*, provides an interesting system for gene analysis since it has a transcriptionally inactive micronucleus and a transcriptionally active macronucleus. The chromosomally located 5S genes are present in both micro- and macronuclei, with about 150 to 300 copies per haploid genome. In the study of Pederson et al.,[537] the chromatin structure of a particular cluster of six 5S genes was analyzed in detail. Defined restriction fragments were isolated from the single copy DNA sequences which flank each side of this gene cluster and these fragments were used to map nuclease cuts in the 5S gene chromatin by the indirect end-labeling technique. The actively transcribed 5S genes of the macronuclei contain DNase I hypersensitive sites at the 5′ side of the transcribed region and within the putative internal promoter. When the rate of transcription of these genes was slowed by starving the cells, the DNase I sensitivity of the internal promoter region was reduced relative to the 5′ site. By contrast, these sites were not found to be hypersensitive to DNase I in the nontranscribed 5S genes of micronuclei. The authors suggest that the degree of nuclease sensitivity of the 5S gene chromatin in growing cell macronuclei indicates that these sequences are free of nucleosomes, possibly as a result of transient displacement by the transcription complex.

The *Physarum* ribosomal genes present a unique situation in that nuclease-resistant particles from the transcribed regions were found to sediment more slowly than particles from the nontranscribed regions (5S vs. 11S).[538-540] These particles, originally called A particles and more recently lexosomes,[335] were found to contain the four core histones as well as other proteins, of which, LP30 and LP32 were present in close to stoichiometric amounts in lexosomes but were absent from the 11S monomers. The core histones were present in both the lexosome and the 11S monomer. Prior et al.[335] showed that the H3 sulfhydryl in lexosomes was reactive toward iodoacetamidofluorescein while that in H3 of 11S monomers is not. Electron microscopic visualization of the two types of particles suggested that the nucleosomes in the lexosome may have undergone a considerable conformational change to form two tetramers separated by about 50 base pairs of DNA. The difference in particle sedimentation, sulfhydryl reactivity, and shape in the electron microscope all suggested a more open structure for the lexosome.

The authors also suggested that the histone-DNA interactions were somewhat weakened in the lexosome, sometimes leading to the isolation of partially histone-depleted particles.[538] This finding indicates that other reports in the literature[497,541,542] of histone-depleted structures may have also been the result of isolation and characterization procedures that were based on bulk chromatin, but which may not have been appropriate for active chromatin. Baer and Rhodes[542] found that RNA polymerase II bound to a subset of nucleosome core particles. These complexes were found to be partially deficient in H2A and H2B.

Using the chicken embryo $tRNA_2$ lys gene, Wittig and Wittig[543] developed an in vitro system which allows for site-directed assembly of nucleosomes on a defined DNA sequence. The system was used to study how particular phase relationships of nucleosomes on the regulatory gene sequences affected the transcription of a particular gene. Plasmids containing this gene were manipulated in such a way as to produce circular DNA molecules in which different lengths of DNA in the gene were made single stranded. The defined circular DNA constructs were combined with a soluble nucleoprotein fraction and a histone source in a

very concentrated solution. This mixture was then incubated in the presence of deoxynucleoside triphosphates and adenosine triphosphate to allow synthesis of double-stranded DNA in the single-stranded regions. Nucleosomes were assembled on the DNA concomitantly during the incubation. Three complementary methods of mapping the nucleosome positions revealed that one nucleosome was always centered over the midpoint of the single-stranded stretch in the manipulated DNA. An in vitro transcription system was used to assay and compare both the types of transcripts synthesized and their level of production from the various tRNA nucleoprotein template complexes. They found that the nucleosome position does affect the level of tRNA gene transcription. The highest rates of specific transcription occurred on templates in which the 5′ end of the tRNA gene as well as its 5′-flanking sequences were located in the internucleosomal spacer region.

These studies have indicated some of the multiple roles that histones may perform in regulating transcription. Whether these bits of information concerning histone functions are different aspects of one overall mechanism or are different mechanisms in quite different regulatory situations remains to be discovered.

VII. CONCLUDING REMARKS

Histones, as a major component of eucaryotic chromosomes, are involved in several diverse but fundamental processes: replication, transcription, and germ line functions such as meiotic pairing and chromatin repackaging. With these multiple roles to play, it is not surprising that histones are a particularly complex class of proteins. As this review has discussed, these complexities include a wide variety of isoprotein species, very different controls on the biosynthesis of different isoprotein species, and a wide variety of post-translational modifications.

While the structural aspects of histones are now known in some detail, how these different aspects interrelate to create a structure that functions effectively in its multiple roles is still very poorly understood. The covalent linkage of many diverse functional units together in one entity has made chromatin a difficult subject for biochemistry. These difficulties are being overcome by a variety of techniques, and hopefully, more reliable information and insights are being gathered.

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