Sites of in Vivo Phosphorylation of Histone H5†

Michael T. Sung and Elizabeth F. Freedlender*,‡

ABSTRACT: Previous studies have suggested that the phosphorylation and dephosphorylation of histone H5 play an important role in controlling the condensation of avian erythrocyte chromatin. The present work locates in the polypeptide chain the major sites at which H5 is phosphorylated in vivo. The majority of the radioactivity in ^{32}P-labeled H5 is clustered in two regions of the molecule. Nearly 50% of the ^{32}P is found in the amino-terminal *N*-bromosuccinimide (NBS) peptide (residues 1–28); the remainder is confined to three phosphopeptides arising from the C-terminal half of the molecule (residues 100–200). All phosphopeptides are found in a tryptic digest of monophosphorylated H5, indicating that phosphorylation of a given site is a random event. Automatic Edman degradation of the amino-terminal fragment shows that the radioactivity is equally divided between serines at positions 3 and 7. The C-terminal phosphorylated tryptic peptides share some features with the C-terminal phosphorylation sites in H1. If, as has been postulated, the sites of phosphorylation are in or near DNA combining regions, then H5 may have two DNA combining sites. The location of the phosphorylation sites is discussed in relation to a possible mechanism for controlling chromatin condensation.

During avian erythropoiesis, the erythrocyte specific histone H5 gradually accumulates in the nucleus. Metabolic studies have indicated that H5 is phosphorylated immediately after its synthesis and subsequently becomes dephosphorylated as the erythrocyte matures (Sung, 1977). The timing of dephosphorylation appears to correlate well with genomic inactivation and chromatin condensation. In vitro circular dichroism studies show that the highly phosphorylated H5 is much less efficient in causing conformational changes in DNA than the unmodified form (Wagner et al., 1977). The location of these phosphates along the polypeptide chain is central to the understanding of the biological role of H5.

In the ubiquitous histone classes, postsynthetic modifications are confined to a few specific residues. The modified residues are located in the highly basic regions which are postulated to be the DNA combining sites (Dixon et al., 1975). Present data indicate that H5 is also phosphorylated in the highly basic C-terminal region but in addition contains phosphates in the nonbasic N-terminal region.

Methods

Preparation of ^{32}P-Labeled H5. Anemia was induced in Leghorn chickens with 2-acetylphenylhydrazine as described by Sung et al. (1977). Bone marrow cell suspensions from these chickens were incubated in vitro with ^{32}P-labeled inorganic phosphate (1–2 mCi/mL) for 1.5 h at 40 °C (Sung et al., 1977). Following incubation, nuclei were isolated as described previously (Sung et al., 1977) and total histones were extracted with 0.2 N HCl. The HCl extract was desalted on a Sephadex G-25 column, eluted with 0.01 M HCl, and made 5% in perchloric acid to precipitate H2a, H2b, H3, and H4. After centrifugation the supernatant was again desalted and freeze-dried. H5 was separated from H1 by Bio-Gel P-100 column chromatography (2.5 × 100 cm; Sung, 1977).

Chemical and Enzymatic Cleavages of ^{32}P-Labeled H5. (i) CNBr Cleavage at Met-31. Ten milligrams of ^{32}P-labeled H5 was dissolved in 2 mL of 70% formic acid and reacted with 420 mg of CNBr at room temperature for 24 h (Gross, 1967). The solution was freeze-dried and the fragments were separated by chromatography on a Sephadex G-75 column (2.0 × 200 cm) eluted with 0.01 M HCl, 0.5 M NaCl. The column fractions were monitored for absorbance at 230 nm and for ^{32}P radioactivity by scintillation counting. The peptides were desalted on Sephadex G-25 and lyophilized.

(ii) NBS[1] Cleavage. The ^{32}P-labeled H5 or its C-terminal CNBr fragment (residue 31–200) was oxidatively cleaved at tyrosines with *N*-bromosuccinimide (NBS) as described by Bustin & Cole (1969). The peptide fragments were separated on the same Sephadex G-75 column described above.

(iii) Chymotrypsin Cleavage. Chymotrypsin (Worthington premium grade) was dissolved in 0.001 N HCl. The conditions for limited digestion were established by kinetic studies on the hydrolysis of H5 at room temperature with an enzyme to substrate ratio of 1/5000 (w/w). The extent of hydrolysis was analyzed on 10% polyacrylamide gels (Panyim & Chalkley, 1969); 15 min of digestion was selected as the optimal time. ^{32}P-labeled NBS peptide was cleaved under these conditions and the peptides were separated on the Sephadex G-75 column described above.

Tryptic Phosphopeptides. (i) N Terminal. A mixture of ^{32}P-labeled, N-terminal CNBr fragment (1–31) and the corresponding unlabeled carrier (3 μmol) in 0.1 M NH_4HCO_3 was digested with β-trypsin at an enzyme to substrate ratio of 1/50 (w/w) for 7 h at 37 °C. At the end of the reaction, the digest was acidified, lyophilized, and redissolved in 0.01 N HCl. The peptides were chromatographed on a Bio-Gel P-2 column (2.0 × 100 cm) in 0.01 N HCl and 4-mL fractions were collected. The ^{32}P-labeled, N-terminal peptide eluted in a single peak. The peptide was lyophilized and dissolved in distilled water. After adjusting the pH of the peptide solution to 2.0, it was applied to a Dowex 50-X4 column (0.9 × 60 cm) in the pyridinium form. The column was maintained at 40 °C

† From the Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901 (M.T.S.), and The Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706 (E.F.F.). *Received October 24, 1977.* This is paper 3 in the series "Phosphorylation and Dephosphorylation of Histone H5," and paper 2180 from the Laboratory of Genetics. This work was supported by grants from the American Cancer Society (NP-123 and NO-160) and The National Institutes of Health (PHS HL 20300).

‡ Present address: Cancer Research Center, University of North Carolina, Chapel Hill, North Carolina 27514.

[1] Abbreviation used: NBS, *N*-bromosuccinimide.

0006-2960/78/0417-1884$01.00/0

TABLE I: Amino Acid Sequence of H5.[a]

```
1          10          Q          20
T-E-S-L-V-L-S-P-A-P-A-K-P-K-  -V-K-A-S-R-
                           R

              NBS  CNBr
                ↓ 30 ↓                40
R-S-A-S-H-P-T-Y-S-E-M-I-A-A-A-I-R-A-E-K-

                        NBS       NBS
                  50     ↓          ↓ 60
S-R-G-G-S-S-R-Q-S-I-Q-K-Y-I-K-S-H-Y-K-V-

                    70                  80
G-H-N-A-D-L-Q-I-K-L-S-I-R-R-L-L-A-A-G-V-

                      Chymotrypsin
                    90    ↓             100
L-K-Q-T-K-G-V-G-A-S-G-S-F-R-L-A-K-S-D-K-

                    110                 120
A-K-R-S-P-G-K-K-K-K-A-V-R-R-&-T-&-P-K-K
```

[a] The amino acids are indicated by the acceptable one-letter code [(1968) *Biochemistry 7*, 2703]; & indicates that whether the residue is serine or alanine was not determined.

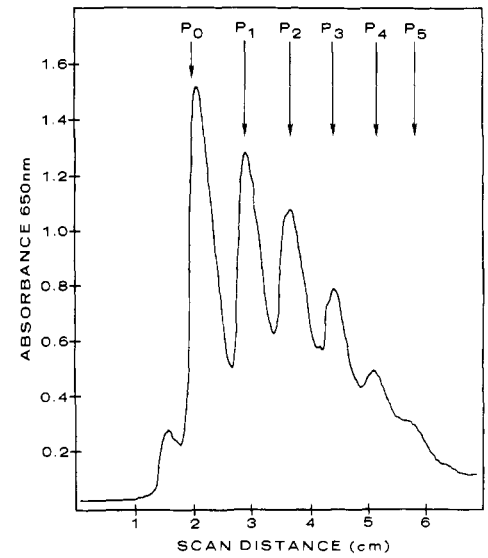

FIGURE 1: Phosphorylated components of histone H5. A sample of anemic bone marrow H5 was electrophoresed in an urea–aluminum lactate gel and the protein components were demonstrated by amido black 10B staining. Electrophoresis was from left to right. The gel was scanned at 650 nm in a Gilford spectrophotometer equipped with a linear transport scanner.

with a constant-temperature circulator bath. The column was developed with a gradient of pyridinium acetate from pH 2.85 (0.05 N) to pH 3.1 (0.3 N) to pH 5.0 (2.0 N). Reservoirs containing 200 mL of each buffer were connected in series by siphons. The pH 2.85 and pH 3.1 reservoirs were continuously mixed with magnetic stirrers. Aliquots of 0.3 mL were removed from each fraction, dried on filter pads, and counted in toluene scintillation fluid.

(ii) C Terminal. Nonradioactive carrier chymotryptic fragment (residues 94–200) was prepared either by exhaustive NBS cleavage followed by limited chymotrypsin digestion or by limited chymotrypsin cleavage of intact H5 molecule. ^{32}P-labeled chymotryptic fragments together with the carrier fragments were digested with trypsin, separated on Bio-Gel P2, and finally chromatographed on Dowex 50-X4 as above.

High Voltage Paper Electrophoresis of Peptides. High voltage paper electrophoresis was performed with a Gilson electrophoretor (Gilson Medical Electronics). One-dimensional electrophoresis on Whatmann 3MM paper at pH 6.5, 3.6, and 1.9 (Kasper, 1975) was routinely used both analytically to monitor peptide homogeneity and preparatively to purify the peptides further. For peptide purification, the radioactive peptides were identified by autoradiography with Kodak Blue Brand x-ray film. Nonradioactive peptides were located by staining guide strips with cadmium ninhydrin. The peptides of interest were eluted with 30% acetic acid.

Amino-Terminal Sequence Determination. The amino-terminal sequences of the major CNBr and NBS fragments were determined by automatic Edman degradation in an Illitron sequenator using the quantitative procedure described by Smithies et al. (1971). The ^{32}P-labeled residues were identified by evaporating the butyl chloride extracts to dryness, redissolving in Bray's (1960) solution and counting in a liquid scintillation counter.

Starch Gel Electrophoresis and Recovery of the Phosphorylated Histones from Starch Gels. To separate the phosphorylated species of histone H5 (Sung, 1977), 1 mg of ^{32}P-labeled histone H5 was electrophoresed in a 24-cm long and 3-mm thick slab urea–aluminum lactate–starch gel (Sung & Smithies, 1969) at a voltage gradient of 8 V/cm for approximately 14 h. At the end of electrophoresis, the gel was sliced horizontally, surface stained for 1 min with Coomassie brillant blue, and rinsed with distilled water to visualize the stained zones. Protein bands corresponding to P_1, P_2, P_3, P_4, P_5, and P_6 (see Figure 1) were excised and the slices individually positioned in glass tubes (0.9 × 12 cm). The slice was overlaid with 2–3 mm of a solution of 10% acrylamide, 0.067% bisacrylamide, 0.5% *N,N,N,N*-tetramethylethylenediamine, 0.9% acetic acid, and 0.063% ammonium persulfate. After the acrylamide gel had polymerized, a small section of dialysis tubing was fastened securely to the top of the gel tube with a rubber band. The dialysis bag was filled with 0.9% acetic acid and the assembly placed between electrophoretic chambers containing the same solution. The ^{32}P-labeled proteins in the gels were electrophoretically transferred to the dialysis bag; the protein solution was removed from the bag and lyophilized.

Results

Histone H5 from anemic bone marrow contains multiple phosphorylated species. Figure 1 shows the staining pattern of H5 electrophoresed in a urea–aluminum lactate–starch gel. Five bands are clearly visible. The most rapidly migrating band is the unmodified H5 and the remaining bands represent phosphorylated species each differing by one phosphate (Sung et al., 1977). The gel scan shows that approximately 70% of the H5 is phosphorylated. While this gel indicates a minimum of four major phosphorylation sites on the molecule, as many as nine phosphospecies have been observed in ^{32}P-labeling studies (Sung, 1977). The fact that a smaller number of phosphospecies are observed in the stained gel suggests that there may be major and minor sites of phosphorylation.

Regional Distribution of ^{32}P Label in H5. In order to locate the phosphorylated species, a variety of chemical and enzymatic cleavages were performed. Table I gives the H5 sequence determined to date and indicates the positions of the cleavages to be discussed. The amino-terminal sequence of H5 residues (1–31) was determined by Greenaway & Murray (1971). Sautiere et al. (1976) extended the sequence to residue 110.

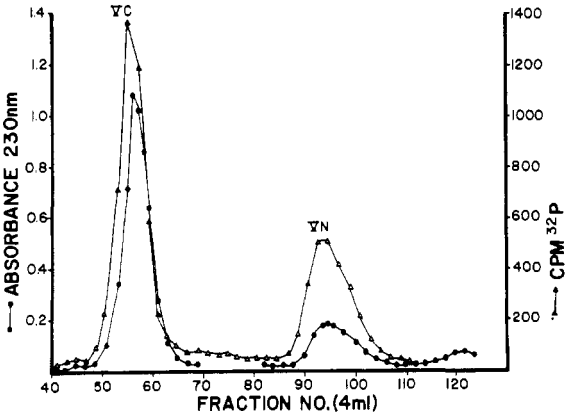

FIGURE 2: Separation of CNBr cleaved ^{32}P-labeled H5 on Sephadex G-75. A sample of ^{32}P-labeled H5 (0.5 μmol) was cleaved at methionine-31 with CNBr. The peptide fragments were separated on a Sephadex G-75 column (2.5 × 150 cm) eluted with 0.01 M HCl, 0.5 N NaCl. The peptides were detected by absorbance at 230 nm and the radioactivity in the peptides was assayed by drying 200 μL of the column fractions on scintillation pads (3MM filter papers) for liquid scintillation counting.

We have confirmed these sequences and extended the data to residue 120. The details of the sequence determination will be published separately.

To more precisely locate the sites of phosphorylation, ^{32}P-labeled H5 was subjected to CNBr cleavage and the resulting peptides were separated. There is a single methionyl residue at position 31 of H5 (Greenaway & Murray, 1971; Sautiere et al., 1976), and as expected two peptides are obtained (Figure 2). The peptides have been identified by molecular weight and amino-terminal sequence. The larger peptide (C-terminal) is comprised of residues 32–200, while the small peptide (N-terminal) represents residues 1–31. As shown in Figure 3, the N-terminal peptide contains approximately 40% of the ^{32}P radioactivity.

Figure 3 (top) shows the peptide fragments arising from NBS cleavage of H5 at the tyrosine residues. The three tyrosines are located in positions 28, 53, and 58 (Sautiere et al., 1976) and the elution "volumes" of these peptides are consistent with the predicted molecular weights. In addition, the peptides have the expected amino-terminal sequences (data not shown). The distribution of ^{32}P radioactivity does not coincide exactly with the optical density profile. Peptides 1–28 and 1–53 together contain nearly 50% of the radioactivity, peptide V (59–200) contains the other 50% of the radioactivity, and peptide V (25–53) contains little, if any, ^{32}P. This distribution is confirmed by the pattern obtained when the C-terminal CNBr peptide (see Figure 2, VC) is treated with NBS (Figure 3, center). Peptide V (32–53) contains no significant counts while nearly all the label is found in peptide V (59–200). The small amount of labeling eluting at the positions of V (1–53) and V (1–28) can be accounted for by the incomplete cleavage in the CNBr reaction. There are no ^{32}P counts at the end of the chromatogram in the region where the short peptide 54–58 elutes; this indicates that the serine at position 55 is not phosphorylated. The combined data indicate that 50% of the ^{32}P label is confined to residues 1–28, while the remainder is located in residues 59–200.

To better locate the C-terminal ^{32}P radioactivity, peptide V (59–200) was cleaved with chymotrypsin. Limited chymotryptic digestion produces two peptides, V (94–200) and V (59–93). All of the label occurs in peptide V (94–200); the identity of this peptide has been established by amino-terminal sequence and molecular weight.

Amino-Terminal Sites of Phosphorylation. In order to locate the ^{32}P-labeled serines in H5 more precisely, the distribution of ^{32}P label in the tryptic peptides was examined by high voltage paper electrophoresis at pH 3.6 followed by autoradiography. Figure 4 shows an autoradiogram of the tryptic peptides of intact H5 (lane 1) and its carboxyl-terminal (lane 2) and amino-terminal (lane 3) CNBr fragments. Only four major radioactive peptides are visible in the digest of intact H5. Peptide T2 clearly arises from the amino-terminal portion of the molecule since it is the only one obtained in the digest of the amino terminal CNBr fragment. When T2 was purified as described in Methods, amino acid analysis demonstrated the following composition: Glu_1, Thr_1, Val_1, Ser_2, Ala_2, Lys_2, Leu_2, Pro_3 (Table II). Gly-0.81 and Ala-0.49 were excluded on the basis of the known amino acid sequence for the N-terminal end of the H5 molecule (Table I) and the fact that Gly and Ala are frequent contaminants when the paper is eluted with 30% acetic acid as in this case. This suggests that T2 is comprised of the amino-terminal residues 1–14. This peptide contains three potential sites of phosphorylation (Thr-1, Ser-3, and Ser-7). The precise location of the ^{32}P has been determined by automatic Edman degradation of unseparated H5 CNBr fragments. The threonines and serines in the two fragments do not occur at the same degradation step. The counts obtained at each step are shown in Figure 5. This clearly shows that serine-3 and -7 are heavily labeled with [^{32}P]phosphate. The repetitive yield between steps 3 and 7 indicates that the two

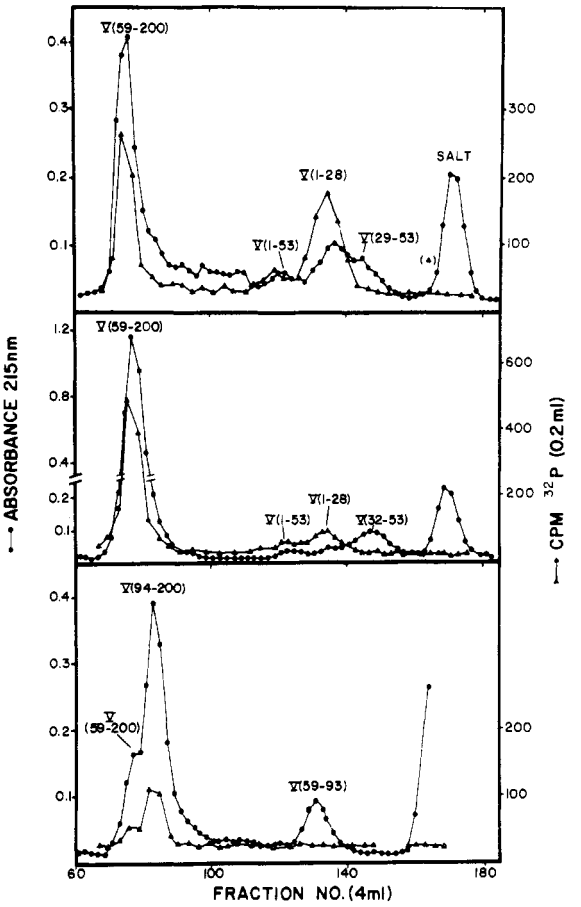

FIGURE 3: Distribution of ^{32}P label in the H5 molecule. Peptides were separated on a Sephadex G-75 column (2 × 200 cm) eluted with 0.1 M HCl, 0.5 M NaCl. The absorbance at 230 nm and radioactivity in the column fractions (4.2 mL) were determined as in Figure 2. Upper panel: ^{32}P-labeled H5 (3 mg) cleaved with *N*-bromosuccinimide. Middle panel: 6 mg of ^{32}P-labeled C-terminal CNBr fragment (Figure 2, VC) cleaved with NBS. Bottom panel: 5 mg of NBS V (59–200) fragment subjected to limited chymotrypsin digestion.

TABLE II: Amino Acid Compositions of Tryptic Phosphopeptides.

Amino acids	N-terminal phosphopeptide T2		C-terminal phosphopeptide T4		C-terminal phosphopeptide $T1_{major}$		C-terminal phosphopeptide $T1_{minor}$		C-terminal phosphopeptide T3	
	Nmol	Residues[a]	Nmol	Residues[a]	Nmol	Residues[a]	Nmol	Residues[a]	Nmol	Residues[a]
Lys	15.9	2 (1.59)	10.17	2 (1.96)	25.65	1 (0.94)	16.41	1 (1.04)	4.5	1 (0.90)
Arg									3.7	1 (0.74)
Thr	9.27	1 (0.93)								
Ser	20.15	2 (2.01)	5.21	1 (1.0)	24.92	1 (0.93)	11.97	1 (0.80)	4.52	1 (0.90)
Glu	13.6	1 (1.36)								
Pro	32.6	3 (3.26)	6.53	1 (1.25)	34.8	1 (1.30)	19.6	1 (1.24)	5.72	1 (1.16)
Gly	8.73	0 (0.87)	4.20	1 (0.81)	26.6	1 (1.00)	6.9	0 (0.43)	2.81	0 (0.56)
Ala	24.9	2 (2.49)	1.72	0 (0.33)	8.12	0 (0.31)	15.8	1 (1.00)	5.21	1 (1.04)
Val	11.9	1 (1.19)								
Leu	20.7	2 (2.07)								

[a] Gly and Ala are frequent contaminants in peptides eluted from paper with 30% acetic acid. For peptide T_2 the values for Gly and Arg were rounded down in light of the known sequence. For the other peptides the values were rounded up if greater than 0.7.

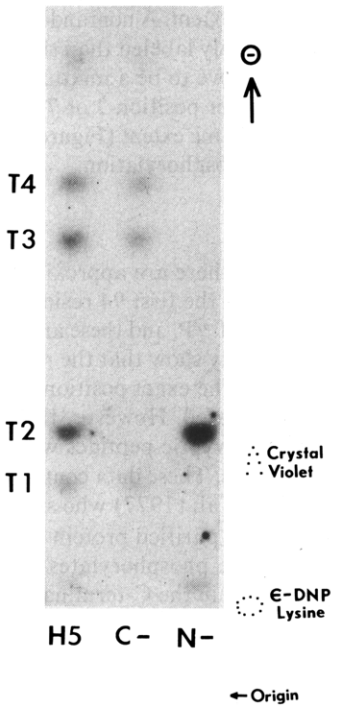

FIGURE 4: Autoradiogram. High voltage electrophoresis of the ^{32}P-labeled tryptic peptides from (a) total H5, (b) CNBr N-terminal fragment (residues 1–31), (c) CNBr C-terminal fragment (residues 32–200). The peptides were electrophoresed at pH 3.6 on Whatman 3 mm paper for 7 h at 15 V/cm. After electrophoresis the paper was dried and the radioactive peptides were detected by autoradiography. Mobilities of phosphopeptides are as follows (all with respect to the dye marker Orange G): T1 (μ = +0.174); T2 (μ = +0.24); T3 (μ = +0.49); T4 (μ = +0.57).

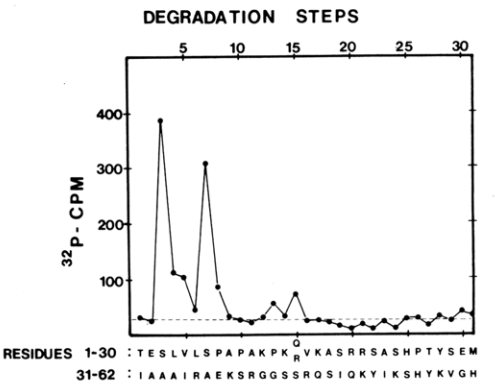

FIGURE 5: Amino-terminal sites of phosphorylation in H5. Automatic Edman degradations of unseparated ^{32}P-labeled CNBr fragments. The counts recovered at each step are plotted against the number of cycles. The expected sequences for the two CNBr fragments are given below.

serines are labeled to the same specific activity. The minor amounts of radioactivity at steps 13 and 15 are probably not phosphorylated serines. There is no serine in either peptide at degradation step 13. The counts at position 15 might represent phosphorylation at Ser-46. However, if this were true, it must have a much lower specific activity than the serines at 3 and 7 since 200 cpm would be expected if the specific activities were the same. Furthermore, there is no carry-over of counts in the subsequent step as would be expected if a phosphorylated serine were present in the sequence.

The mobility of T2 at pH 3.6 ($\mu^{\text{Orange G}} = +0.24$) suggests that the peptide is monophosphorylated at either serine-3 or -7. Indeed, there is a neutral minor phosphopeptide ($\mu^{\text{Orange G}} = 0$) more acidic than T2 which is visible in the tryptic digest of CNBr N-terminal fragment (Figure 4); this may very well be the same tryptic peptide as T2 but phosphorylated at both serine-3 and -7. The peptide yield is too low to permit further characterization and verification.

C-Terminal Phosphopeptides. Figure 4 also shows that the major phosphopeptides T1, T3, and T4 are all present in the CNBr C-terminal fragment. Together with the results shown in Figure 3, this indicates that the phosphorylation sites are beyond residue 94 in the H5 molecule. We attempted to locate the sites in the chymotryptic carboxyl fragment in the protein sequencer. For 30 degradation steps no significant ^{32}P counts were released from the peptide fragment. This suggests that the sites are located further than residue 120 in the carboxyl-half of the molecule.

T_3 and T_4 have been isolated in low yield by the purification procedures described in Methods. The amino acid compositions of T4 (Lys_2, $Ser_1(PO_4)$, Pro_1, Gly_1) and T_3 (Lys_1, Arg_1, $Ser_1(PO_4)$, Pro_1, Ala_1) (Table II) suggest that these two peptides have similar but not identical sequences. Peptide T1 was isolated in good yield; however, provisional amino acid analysis indicated that it was impure. To further purify T1, the sample was treated with *E. coli* alkaline phosphatase and the dephosphorylated peptide subjected to reelectrophoresis at pH 3.6. Quite unexpectedly, two dephosphorylated peptides were obtained and their amino acid compositions analyzed: T1 major (Lys_1, Ser_1, Pro_1, Gly_1) and T1 minor (Lys_1, Ser_1, Pro_1, Ala_1). The amino acid compositions of the C-terminal phosphopeptides suggest that T1 major and T1 minor may arise

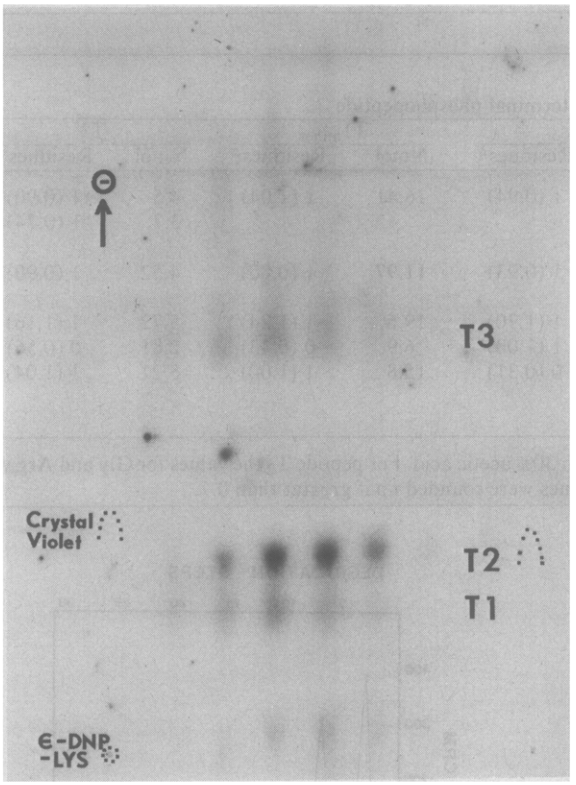

FIGURE 6: Random sites of phosphorylation in H5. The ^{32}P-labeled phosphorylated species P_1, P_2 . . . P_n (see Figure 1) were electrophoretically recovered from urea–aluminum lactate–starch gel as described in Methods. The phosphorylated proteins were each digested with trypsin and subjected to high voltage paper electrophoresis at pH 3.6. The autoradiograph was obtained by exposing Kodak Royal Blue x-ray film for 1 week.

from T4 and T3 by slow tryptic hydrolysis at presumptive Lys-Ser(PO_4) and Arg-Ser(PO_4) bonds, respectively. A precedent for this has been seen in trout testis H1; in this protein two major tryptic phosphopeptides with the sequences Lys-Ser(PO_4)-Pro-Lys and Ser(PO_4)-Pro-Lys (Sung & Dixon, 1970) have been shown to be derived from one thermolytic sequence Ala-Ala-Lys-Lys-Ser(PO_4)-Pro-Lys (Dixon et al., 1975). Assuming the above explanation is correct, the number of major sites of phosphorylation in the carboxyl-terminal portion of H5 is reduced to two. Alternatively, T1 major and T1 minor may be distinct in origin from T3 and T4, thus indicating multiple sites of phosphorylation in homologous and recurring sequences. Again, Dixon et al. (1975) have shown that recurring sequences are very common in the C-terminal half of trout testis H1.

Random vs. Nonrandom Sites of Phosphorylation. We have suggested that various phosphorylated species of H5 represent steps in a pathway of progressive phosphorylation. In this pathway, a single phosphate is added to each histone molecule at each step. Two possibilities exist for the mode of addition of each phosphate: (1) nonrandom phosphorylation—at each step a serine at a particular position in the sequence is phosphorylated by a unique phosphokinase; (2) random phosphorylation—one of several possible sites is phosphorylated. The end result of the two mechanisms is identical, but the nature of the intermediate phosphorylated species is distinct. These two mechanisms can be differentiated by examining the phosphopeptides present in the various phosphorylated species. If phosphorylation occurs nonrandomly, then P_1 will contain only one phosphopeptide while P_n contains n-phosphopeptides. If phosphorylation is random, several phosphopeptides will be present in the population of P_1 molecules.

To determine by which mechanism H5 is phosphorylated, the various phosphorylated species P_1, P_2 . . . P_n were isolated from starch gel and digested with trypsin. The phosphopeptides were identified by high voltage paper electrophoresis. The result shown in Figure 6 strongly favors the random phosphorylation mechanism. The digest of each phosphospecies contains all three of the phosphopeptides (T_1, T_2, and T_3). Thus although P_1 molecules each bear only one phosphate, this phosphate may be located in any of the three typtic peptides. A digest of the population of P_1 molecules, therefore, contains all the phosphopeptides although each individual molecule contains only one. If phosphorylation were nonrandom, the P_1 molecules should contain only one phosphopeptide representing the unique site of the first phosphorylation. Further evidence of random phosphorylation comes from the sequence data which shows that the serines at positions 3 and 7 are phosphorylated to the same extent. A nonrandom mechanism would produce one more heavily labeled than the other. In addition, T2 has been shown above to be a mixture of peptides monophosphorylated at either position 3 or 7. These two sites are phosphorylated to the same extent (Figure 5) providing further evidence of random phosphorylation.

Discussion

In the H5 molecule there are approximately 24 serines of which 14 are present in the first 94 residues. Only 2 of these 15 show incorporation of ^{32}P, and these are located at positions 3 and 7. The data clearly show that the region 8–94 is devoid of labeled phosphate. The exact positions for the C-terminal sites have not been located. However, they appear to be restricted to two sets of tryptic peptides which come from the C-terminal 100 residues. These data contrast sharply with the results of Kurochkin et al. (1977) who studied in vitro phosphorylation of H5 by a purified protein kinase isolated from pig brain. This enzyme phosphorylates three serines in the region 45 to 94 and one in the C-terminal portion of H5. The difference between the two sets of data probably reflects the difference in specificity between the pig brain and erythrocyte enzymes.

Our data show that 50% of the ^{32}P label is equally distributed between serine-3 and -7, while the remaining 50% is located in two sets of related tryptic peptides in the C-terminal end of the molecule. This suggests four major sites of phosphorylation, in agreement with the number of phosphorylated species visible in stained gels. The additional five sites detected in labeling studies (Sung, 1977) may be minor sites which are not detected in the present study.

Histones H2a, H2b, H3, and H4 each contain a single phosphoserine, which is located within the N-terminal 30 residues. By contrast, H1 has multiple sites of phosphorylation. In nondividing cells, about 1% of the H1 molecules are phosphorylated at serine-38. In dividing cells, the degree of H1 phosphorylation changes during the cell cycle. In early G1 nearly all the H1 is unphosphorylated while during mitosis 100% of the H1 molecules are phosphorylated at 1–4 sites (Gurley et al., 1975; Balhorn et al., 1975). In trout H1 one of these sites is Ser-156, and the others are probably at Ser-172 and -196 (Dixon et al., 1975). The amino-terminal portion of H1 in trout testis cells does not appear to be heavily phosphorylated. On the other hand, phosphorylation of the amino-terminal fragment of H1 in CHO and HeLa cells has been shown to occur in the mitotic phase of the cell cycle (Hohmann

et al., 1976; Ajiro et al., 1976). Langan (1976) has reported that this site is at Thr-16 of the H1 molecule.

H1 and H5 differ from the other major histones in size, stoichiometry, polymorphism, ease of dissociation from DNA, and location on the chromatin. Both proteins show an unusual distribution of amino acids: extremely basic at the C-terminal half of the molecule, hydrophobic in the middle, and slightly basic at the amino end (Bustin & Cole, 1970; Jones et al., 1974; Sautiere et al., 1976; Yaguchi et al., 1977). Additional evidence for the similarity of H1 and H5 comes from the extensive sequence homology in the amino half of the two proteins (Strickland et al., 1976; Yaguchi et al., 1977; Hurley, personal communication). Spectroscopic studies have also indicated that H5 and H1 have similar secondary structures (Crane-Robinson et al., 1976). Hartman et al. (1977) have defined three conformational domains in the H1 molecule: a globular central domain consisting of residues 42–120 and two random coil domains at both ends of the molecule. On the basis of sequence homology alignments, residues 24–100 in H5 may also be globular while residues 1–22 and 110–C terminus are random coils. Further, in terms of metabolism both histones are phosphorylated at multiple sites and are neither ϵ-aminoacetylated or methylated (Sung et al., 1977). In sum these relations suggest a common evolutionary history for the two proteins. H5 may in fact be a highly differentiated H1 histone whose sole function is to maintain the highly repressed state in avian erythrocyte chromatin.

The hypothesis that H1 may play a role in the condensation of chromatin has been suggested for some time (Littau et al., 1965) and has recently received considerable experimental support (Bradbury et al., 1973; Lewis et al., 1976; Thoma & Koller, 1977; Hsiang & Cole, 1977; Vengerov & Popenko, 1977; Griffith & Christiansen, 1978). A similar role for H5 is suggested by the observation that extraction of H5 appears to loosen condensed chromatin fibrils as judged by electron microscopy (Brasch et al., 1972). Evidence for the physiological relevance of this phenomenon comes from the observation by Appels et al. (1974) that loss of H5 correlates with the reactivation and decondensation of chromatin in chick nuclei present in chick/HeLa heterokaryons.

It has been postulated that the basic region of histones binds to DNA. The similarity of the C-terminal regions of H5 and H1 may facilitate the replacement of H1 by H5 during erythrocyte maturation (Sotirov & Johns, 1972; Sung, 1977). The presence of phosphates in the amino-terminal end of H5 is reminiscent of the phosphate location in the DNA combining region of the other four histones. The absence of phosphates in the central globular domain is significant since this region is thought not to be involved in DNA binding (Bradbury et al., 1974; Worcel & Benyajati, 1977). Indeed, if phosphorylation occurs in DNA combining sites, the clustering of the sites of phosphorylation at the two ends of the H5 molecule suggests that H5 has two DNA combining sites. Two combining sites may allow H5 to cross-link DNA molecules causing condensation of the chromatin.

Phosphorylation of the N-terminal region may be important in the condensation of erythrocyte chromatin. H5 differs from the four histones (H4, H2a, H2b, H3) in having two phosphates and no adjacent basic residues in the amino terminal region. Thus, phosphorylation of both serine-3 and -7 produces an extremely acidic region at the N terminus. Perhaps electrostatic repulsions between the phosphorylated amino end and the DNA prevent premature condensation while allowing the C-terminal end of the molecule to bind to DNA. Removal of the negatively charged phosphates would relieve this repulsion and lead to condensation. This is consistent with the observation that H5 in the erythroblast is highly phosphorylated and the chromatin is not condensed while the level of phosphorylation of H5 decreases and the chromatin becomes more condensed as the erythrocyte matures (Sung, 1977).

Acknowledgment

The authors wish to thank Dr. Oliver Smithies for his constant encouragement and for providing the sequencing facilities.

References

Ajiro, K., Borun, T. W., & Cohen, L.H. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1623.

Appels, R., Bolund, L., & Ringartz, N. R. (1974) *J. Mol. Biol. 87,* 339.

Balhorn, R., Jackson, V., Granner, D., & Chalkley, R. (1975) *Biochemistry 14,* 2504.

Bradbury, E. M., Carpenter, B. G., & Rattle, H. W. E. (1973) *Nature (London) 241,* 123.

Bradbury, E. M., Ingles, R. J., & Matthews, H. R. (1974) *Nature (London) 247,* 57.

Brasch, K., Setterfield, G., & Neelin, J. M. (1972) *Exp. Cell Res. 30,*

Bray, G. A. (1960) *Anal. Biochem. 1,* 279.

Bustin, M., & Cole, R. D. (1969) *J. Biol. Chem. 244,* 5291.

Bustin, M., & Cole, R. D. (1970) *J. Biol. Chem. 245,* 1458.

Crane-Robinson, C., Danby, S. E., Bradbury, E. N., Garel, A., Kovacs, A. N., Champagne, M., & Daune, M. (1976) *Eur. J. Biochem. 67,* 379.

Dixon, G. H., Candido, E. P. M., Honda, B. M., Louie, A. J., MacLeod, A. R., & Sung, M. T. (1975) *CIBA Foundation Symp. 28,* 229.

Greenaway, P. J., & Murray, K. (1971) *Nature (London), New Biol. 229,* 233.

Griffith, J. D., & Christiansen, G. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42* (in press).

Gross, E. (1967) *Methods Enzymol. 11,* 238.

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

Hartman, P. G., Chapman, G. E., Moss, T., & Bradbury, E. M. (1977) *Eur. J. Biochem. 77,* 45.

Hohmann, P., Tobey, R. A., & Gurley, L. R. (1976) *J. Biol. Chem. 251,* 3685.

Hsiang, M. W., & Cole, R. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 4852.

Jones, G. M. T., Rall, S. C., & Cole, R. D. (1974) *J. Biol. Chem. 249,* 2548.

Kasper, C. B. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) p. 158, Springer-Verlag, New York, N.Y.

Kurochkin, S. N., Andreeva, N. B., Gasaryan, K. A., & Severin, E. S. (1977) *FEBS Lett. 76,* 112.

Langan, T. A. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1623.

Lewis, E. A., DeBuysere, M. S., & Rees, A. W. (1976) *Biochemistry 15,* 186.

Littau, V. C., Burdick, C. J., Allfrey, V. G., & Mirsky, A. E. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54,* 1204.

Louie, A. J., & Dixon, G. H. (1973) *Nature (London), New Biol. 243,* 164.

Panyim, S., & Chalkley, R. (1969) *Biochemistry 8,* 3972.

Sautiere, P., Briand, G., Kmjecik, D., Loy, O., Biserte, G., Garver, A., & Champagne, M. (1976) *FEBS Lett. 63,* 164.

Smithies, O., Gibson, D., Fanning, E. M., Goodfliesch, R. M., Gilman, J. G., & Ballantyne, D. L. (1971) *Biochemistry 10,*

4912.
Sotirov, N., & Johns, E. W. (1972) *Exp. Cell Res. 73,* 13.
Strickland, W. N., Schaller, H., Strickland, M., & Van Holt, C. (1976) *FEBS Lett. 66,* 322.
Sung, M. T. (1977) *Biochemistry 16,* 286.
Sung, M. T., & Dixon, G. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67,* 1616.
Sung, M. T., & Smithies, O. (1969) *Biopolymers 7,* 39.
Sung, M. T., Harford, J., Bundman, M., & Vidalakas, G. (1977) *Biochemistry 16,* 279.
Thoma, F., & Koller, Th. (1977) *Cell 12,* 101.
Vengarov, Y. Y., & Popenko, V. I. (1977) *Nucleic Acids Res. 4,* 3280.
Wagner, T., Harford, J., Serra, M., Vandergrift, V., & Sung, M. T. (1977) *Biochemistry 16,* 289 (Appendix).
Worcel, A., & Benyajati, S. (1977) *Cell 12,* 83.
Yaguchi, M., Roy, C., Dove, M., & Seligy, V. (1977) *Biochem. Biophys. Res. Commun. 76,* 100.

Bleomycin-Specific Fragmentation of Double-Stranded DNA†

R. Stephen Lloyd,‡ Charles W. Haidle,* and Donald L. Robberson

ABSTRACT: Brief exposure of covalently closed circular duplex PM2 DNA to low concentrations of the clinical bleomycin mixture (Blenoxane) resulted in specific fragmentation of the genome that does not depend on the presence of superhelical turns. The double-strand breaks are in fact produced at several discrete sites on the PM2 genome but frequently occurring near the *Hpa*II restriction endonuclease cleavage site. Initial rates of formation of nicked circular and linear duplex PM2 DNAs are reduced to different extents as the ionic strength of the reaction is increased. Increasing ionic strength is most effective in reducing the initial rate and overall yield of apparent double-strand scissions compared with single-strand scissions in the bleomycin-treated PM2 DNA.

The bleomycins are a group of glycopeptide antibiotics produced by *Streptomyces verticillus* (Umezawa et al., 1966) and have been used in clinical treatment of human malignancies (Ichikawa, 1970; Shastri et al., 1971; Blum et al., 1973). An extensive review of the action of bleomycin has been published recently (Müller & Zahn, 1977). Although the physiological basis of drug action has not yet been defined, the antibiotic mixture has been shown to cause extensive single-strand breakage of DNA, in vitro, when the reaction products are assayed by velocity sedimentation in alkaline sucrose gradients (Suzuki et al., 1969; Haidle, 1971). It has also been shown that DNA strand scissions are produced more efficiently with single-stranded compared with double-stranded DNAs (Suzuki et al., 1969; Haidle, 1971). Furthermore, shifts in the velocity sedimentation profiles at neutral pH suggested the possibility that bleomycins also produce double-strand breaks in duplex DNA (Haidle, 1971). This earlier study did not, however, determine if the putative double-strand breaks may not have arisen by an accumulation of single-strand scissions. This latter consideration is particularly significant since high concentrations of bleomycin (more than approximately 1.9 mol of bleomycin per mol of DNA phosphate) and extensive reaction conditions had been employed (Haidle, 1971). Double-strand breaks also occur in DNA extracted from bleomycin-treated mammalian cells, but again an accumulation of single-strand scissions could have accounted for these observations (Saito & Andoh, 1973; Byfield et al., 1976).

Recently, a study of bleomycin treated T2 and ColEl DNAs has demonstrated that double-strand breaks are produced in proportion to the number of single-strand breaks accumulated, but are much more frequent than the number expected simply from random coincidence of single-strand breaks (Povirk et al., 1977). Regardless of the mechanism of production of the double-strand breaks, it was deemed important to determine whether there were specific sites on the DNA molecule at which these breaks occurred and to determine some of the properties of the chemical reaction. Possible approaches in defining the modes of action for different component bleomycins which are applied in mixture for clinical chemotherapy may be indicated by the findings of these studies.

Materials and Methods

Preparation of PM2 DNAs. Covalently closed circular PM2 form I DNA (Espejo & Canelo, 1968) was isolated by extraction of purified bacteriophage according to a modification (Strong & Hewitt, 1975) of the procedure described by Salditt et al. (1972). The closed circular DNA was isolated from the lower band in buoyant CsCl[1] gradients containing saturating levels of ethidium bromide (Radloff et al., 1967). After removal of ethidium bromide (EthBr) by extraction with NaCl-saturated isopropyl alcohol, the DNA samples were dialyzed against several changes of 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and stored at 4 °C. Covalent closed circular PM2 form I° DNA (relaxed form I DNA) was prepared by treatment of purified form 1 DNA with the calf thymus nicking-closing enzyme (Pulleyblank & Morgan, 1974; Hancock, 1974; Germond et al., 1975) using the reaction conditions described by Champoux & McConaughy (1976). The form I° DNA was isolated from the lower band in EthBr–CsCl gradients as described above. The EthBr was

† From the Department of Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030. *Received December 6, 1977.* This work was supported in part by Grant No. CA 13246 and CA 16527 awarded by the National Cancer Institute, Department of Health, Education, and Welfare, and Grant No. G-441 awarded by the Robert A. Welch Foundation.
‡ A predoctoral fellow with the Robert A. Welch Foundation.

[1] Abbreviations used: CsCl, cesium chloride; EthBr, ethidium bromide; EDTA, ethylenedinitrilotetraacetic acid; SSC, standard saline citrate.

0006-2960/78/0417-1890$01.00/0