A Low-Electrophoretic-Mobility H1 Histone Subfraction from Kirkman-Robbins Hamster Hepatoma

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ABSTRACT

A protein showing lower electrophoretic mobility in acidic urea polyacrylamide gels than did the usual histone H1 subfractions has been detected among the H1 histones extracted from chromatin of a transplantable hamster hepatoma, originally induced by Kirkman and Robbins. It was proved to be a true H1 histone subfraction. It differs from the remaining ones by the total chain length, amino acid composition, and isoelectric point value. It is not a phosphorylated or phosphorybyslated metabolic form of another subfraction. Its proteolytic degradation products (obtained by thrombin and trypsin digestion) closely resembled those obtained from other H1 subfractions. The investigated hepatoma seems to provide an interesting model of neoplastic cells showing a distinct difference in histone composition from the homologous normal tissue.

INTRODUCTION

The only property common for all cancerous cells is their ability for unlimited growth directed by the unknown mechanisms governing development and regeneration. Numerous facts let us assume that these mechanisms are functioning within the cellular genome. For this reason, a great deal of investigations on the molecular basis of carcinogenesis concentrate upon the composition, structure, and functioning of the constituents of neoplastic cells. Considerable attention has been paid to histones isolated from cancerous cells. General impression left by investigations published in the 1960's and 1970's was the lack of specific constituents and their possible role in carcinogenesis. Some laboratories reported slight differences in the general spectrum of various nonhistone protein groups (35, 36), but their understanding in molecular terms is limited by merely small amounts of these proteins available experimentally so far. One of us (G. M. G.) initiated the analytical comparison of histone fractions in a series of normal and neoplastic tissues. It led to an accidental discovery of an atypical electrophoretic fraction of reduced mobility among the perchloric acid-soluble H1 group isolated from a transplantable hamster tumor. The tumor was originally induced by testosterone administration by Kirkman and Robbins (19).

The reproducible appearance of this protein, the almost constant ratio of the intensity of its band to those of the remaining histone fractions, and its solubility in perchloric acid suggested that it may represent a specific H1 histone subfraction perhaps analogous to some H1 subfractions of decreased mobility reported recently to occur in testicular extracts (6, 21, 28, 29) and supposed to be related to spermatocyte formation (26). Taking into account its absence in the homologous normal tissue, the hamster liver, and the growing interest in the interrelation between the composition of the H1 subfraction set, differentiation, and neoplastic growth (7, 8, 12, 17, 27, 37), we decided to undertake a more detailed study of the nature of this subfraction.

So far, there have not been introduced any unified rules for the nomenclature of H1 subfractions. In order to avoid confusion caused by using the same symbols when describing unusual H1 subfractions from various sources, as for example H1° (28), we named the low-mobility subfraction from Kirkman-Robbins hamster hepatoma 'H1-slow.'

MATERIALS AND METHODS

Transplantation of the Tumor

A Kirkman-Robbins hamster hepatoma was obtained at the 447th passage from the Department of Pathological Anatomy of the Medical School in Wrocław, where it originally arrived from the Chester Beatty Research Institute in London at the 167th passage. For cell line continuation, 0.2 ml of a suspension of mechanically dispersed neoplastic cells taken from an 8-day-old tumor was inoculated s.c. above the axilla of 2 Syrian hamsters. The passages were made every 8 days. For histone isolation experiments, 20 to 30 2-month-old hamsters were inoculated simultaneously, and the neoplastic material was collected on the eighth day after the transplantation. Tumors growing for longer than 8 days, although larger, showed distinct quickly increasing signs of necrosis. Livers of control animals were taken as reference tissue. Looking for a more efficient production of the neoplastic tissue mass, free of necrosis, we switched lastly to symmetrical double injections into both axillas of the animal.

Isolation of the Cellular Nuclei

All steps were performed at 2° in the cold room, and it is specified if otherwise. Three- to 5-g samples of both tumor and liver tissues were cut into small fragments and homogenized in a Potter-Elvejhem homogenizer with a Teflon pestle in 10 volumes of Homogenization Medium A [0.25 m sucrose containing 0.8 mM KH2PO4. 1.8 mM CaCl2. and 0.5 mM phenyl-methanesulfonyl fluoride (pH 6.5)]. The tissue was first homogenized in a small volume of the medium until uniformly dispersed. Then, the rest of the medium was added and homog-
The homogenate was filtered through 4 sheets of cheesecloth and centrifuged at 850 × g for 5 min. The sediment of crude nuclei was resuspended in 10 volumes of Homogenization Medium A, overlayered on 20 volumes of 0.4 M sucrose containing additional components as in Homogenization Medium A, and centrifuged at 850 × g for 5 min. The sediment was washed 4 times in 10 volumes of 1% gum arabic solution, containing additional components as in Homogenization Medium A, overlayered, and centrifuged as above. For final purification, the nuclei were suspended in 10 volumes of Homogenization Medium A, layered over 20 volumes of 0.88 M sucrose containing other components as Homogenization Medium A, and centrifuged at 2000 × g for 10 min. The purity of the final samples of nuclei was checked in a microscopic test using methyl green-pyronine staining technique.

Isolation of the H1 Histone Group

The purified nuclear pellet was washed 5 to 6 times with 6 volumes of 0.14 M NaCl, pH 5.4, and 2 times with 6 volumes of 0.35 M NaCl (13), pH 5.4, both containing 0.5 mM phenyl-methanesulfonfyl fluoride. Each time, it was suspended in the Potter-Elvehjem homogenizer by 40 full pestle shifts, extracted for 20 min with magnetic stirring, and centrifuged at 1100 × g for 30 min. The remaining sediment was extracted in a similar way once with 4 volumes of 0.74 M HClO₄, extracted once with 2 volumes of the same solution, and each time centrifuged at 1100 × g for 30 min (16). The H1 histone group was precipitated from the combined perchloric acid extracts by adding 100% (w/v) trichloroacetic acid solution up to the final concentration of 18% and leaving the sample overnight. The sediment was collected by centrifugation and washed 3 times with acetone and washed 10 volumes of acetone to the supernatant. The precipitate, collected by centrifugation, was washed 3 times with acetone and dried under vacuum.

Electrophoretic Characterization of H1 Subfractions

The composition of the set of subfractions in the total H1 histone was analyzed by polyacrylamide gel electrophoresis according to the method of Panyim and Chalkley (24) in 25-cm-long 2.5 M urea gels using a separation time of 72 hr at 2°C. The gels were stained overnight with Amidoblack 10B and the separation procedure was followed as described by Smith and Stocken (34).

Removal of Phosphorylated Forms of the H1 Subfractions by Enzymatic Dephosphorylation. The phosphorylated molecules of the H1 subfractions were turned into their unmodified forms by digestion with the alkaline phosphatase from Escherichia coli (EC 3.1.311; Worthington Biochemical Corp., Freehold, N.J.) according to the method of Balhorn et al. (4).

Testing for the Presence of ADP-ribosylated Forms. Approximately 0.1% solutions of the total H1 histone and of purified H1-slow in 0.01 M HCl were used for control of UV absorbance spectrum in the range of 250 to 280 nm.

Preparative Separation of H1 Subfractions on Amberlite CG-50 Columns

H1 subfractions were separated according to the method of Kinkade and Cole (18) as described by Bonner et al. (5) and Smerdon et al. (33), slightly modified by using a more flat guanidine hydrochloride linear gradient prepared by mixing 800 ml of 9.3% and 800 ml of 10.8% solutions, both containing 0.1 M phosphate buffer and adjusted to pH 6.8. They were pumped through a 2.5- × 40-cm column (Pharmacia Fine Chemicals, Uppsala, Sweden) at the rate of 8 ml/hr. Four-mI fractions were collected, and their protein content was estimated by measuring the absorbance caused by sample turbidity after trichloroacetic acid addition at 400 nm.

Semipreparative Electrophoretic Separation of the Mixture of H1 Subfractions

Some chromatographic peaks appeared to contain more than one electrophoretic fraction. In order to obtain homogeneous material (giving a single band), the dry chromatographic fractions were dissolved in 0.9 M CH₃COOH plus 15% sucrose to give a concentration of 1 to 2 μg/μl and loaded onto a 1.0-cm (inside diameter), 20-cm-long columns of polycrylamide gel, prepared as for analytical runs. The samples (120 μg protein per column) were run for 24 hr at 190 V at room temperature. The fractions were localized by gentle staining of the gel surface. For that purpose, the gels were immersed for 2 hr in 0.1% amidoblack 10B-7% acetic acid-20% ethanol stain 30 minutes diluted with 0.9 M CH₃COOH. The blue-stained surface circles were clearly visible against the faint background. The blue slices were cut out, and the corresponding ones were pooled together and tightly inserted into 1.1-cm (inside diameter), 20-cm-long glass tubes, closed at the bottom with a 2-cm layer of polymerized gel. The protein was driven electrophoretically into an elution chamber (approximately 10 cm) separated from the electrode vessel by a dialysis membrane. Its content was dialyzed against 0.01 M HCl and concentrated. The proteins were precipitated by adding 10 volumes of acetone, washed, and dried as described above.

Analysis of the Amino Acid Composition of the Subfractions

Electrophoretically homogeneous subfractions were hydrolyzed under vacuum, oxidized, and analyzed in the Jeol JLC-3BC 2 automatic amino acid analyzer according to the methods of Hirs (15) and Moore and Stein (22).

Evaluation of the Molecular Weight of the H1 Subfractions

The approximate values of the molecular weight of individual H1 subfractions were estimated using the sodium dodecyl
sulfate-polyacrylamide gel electrophoresis technique of Pan-
yim and Chalkley (25), performed in principle as described by
Weber and Osborne (38).

**Limited Degradation of the H1 Subfractions by Proteolytic
Activity of Thrombin**

The conditions for proteolytic degradation were those de-
scribed by Chapman et al. (9). The resulting degradation prod-
ucts were separated by electrophoresis and estimated accord-
ing to the same authors.

**Two-Dimensional Separation of Tryptic Peptides**

Purified H1 subfractions were submitted to limited digestion
with trypsin according to the method of Hartman et al. (14).
The resulting peptides were separated in a 2-dimensional chro-
matographic-electrophoretic system of Sherod et al. (31) on
Whatman No. 3MM filter paper. The separated peptides were
localized by the ninyhydrin method or by the phenanthroquinone
method for arginine-containing peptides according to Easley et
al. (11).

**Search for Sugar Residues**

Some electrophoretic separations were used for sugar-spe-
cific staining according to Zacharius et al. (40).

**Determination of Isoelectric Points of H1 Subfractions**

The isoelectric point values of the individual H1 subfractions
and their homogeneity in ampholine-containing polyacrylamide
gels were estimated according to the method of Kopelovich et
al. (20).

**RESULTS**

As in other laboratories, the tumor in our transplantation
experiments revealed an almost 100% transplantability. The
average weight of a single 8-day-old purified tumor tissue was
about 2.2 g. Symmetrical double transplantations allowed us
to increase the yield up to approximately 4.3 g from one animal.

Purified nuclei were isolated with the average yield of 10 to
11 ml of packed wet weight nuclei, starting with 50 g of tumor
tissue. Their purity was evaluated in a microscopic test after
methyl green-pyronine staining, indicating the absence of frag-
ments of cytoplasm on the nuclear surface.

The resulting H1 histone preparations were characterized by
high-selectivity polyacrylamide gel electrophoresis (Chart 1).

In order to exclude the possibility that H1-slow represents a
nonhistone protein fraction of lower mobility known to contam-
inate H1 histone preparations or a metabolic modification of
the H1 subfraction, 3 procedures were introduced. (a) "Protein
P"-like components, if present, were removed by column chro-
matography on DE-52 cellulose (Charts 2 and 3). (b) Phosphorylated H1 histone subfractions were turned into their
usual forms by incubation with Escherichia coli alkaline phos-
phatase. Electrophoretic runs of the same H1 preparation
before and after phosphatase treatment are compared in Chart
4. And (c) UV the spectrum was checked in the 250 to 280-nm
range. If each H1 molecule was loaded with one ADP ribosyl
residue, the A260 of a 0.023% solution should be approxi-
mate 0.150. In fact, the A260 of a 0.1% solution did not
exceed 0.03, a value limiting the number of ADP ribosylated
molecules to less than 1 per 20. Direct measurements of A260
of the H1-slow solution (it had to be purified previously from
some gel-extracted contaminations) definitely exclude the pos-
sibility that H1-slow is an ADP ribosylated H1 molecule.

Larger quantities of individual H1 subfractions, sufficient for
further chemical investigations, were obtained by column chro-
matography of the H1 histone on Amberlite CG-50 or Bio-Rex
70 columns. The typical elution profiles for tumor and liver H1
histone fractionation are shown in Charts 5 and 6. In Charts 5B
and 6B are presented the densitograms of electrophoretic runs
illustrating the subfractional composition of the subsequent
chromatographic peaks. The complete scheme for electropho-
retic identification of the tumor and liver subfractions present
in subsequent peaks, as compared with the standard H1 his-
tone from calf thymus, is shown in Chart 7.

As shown in Charts 5 and 7, the specific low-mobility subfrac-
tion H1-slow present in hepatoma chromatin cannot be sepa-
rated from other H1 subfractions solely by column chromatog-
raphy. Part of this subfraction appears in Peak 1 in an almost
equimolar mixture with the fastest subfraction, H1-4, and the
mixture of H1-slow and H1-4, as possible (up to 120 µg/column). The recovery of purified fractions did not exceed 40%. They were electrophoretically homogeneous, and the mobility of those purified preparations was unchanged (Chart 8). In accordance with the observation of Chrambach et al. (10), they were contaminated to a various degree by some mobile gel components which, however, coprecipitated with

rest is found in Peak II contaminated with 2 other subfractions.

The fact that, accidentally, Peak I contains the slowest and the fastest among the H1 subfractions enabled us to develop a semipreparative electrophoretic procedure for isolation of pure H1-slow. The distance, although remarkable, was too small for a typical preparative gel electrophoresis procedure where mixing of subsequent subfractions would certainly occur in the elution chamber. Therefore, we applied in parallel a large number of analytical columns of larger diameter loaded with as much of the contents of chromatographic Peak I, i.e., the

Chart 3. Comparison of electrophoretic densitograms of the H1 histone preparation from Kirkman-Robbins hepatoma before (a) and after (b) purification on DEAE cellulose DE-52. For electrophoretic conditions, see legend to Chart 1.

Chart 4. Comparison of electrophoretic densitograms of the H1 histone from Kirkman-Robbins hepatoma before (a) and after (b) dephosphorylation. For electrophoretic conditions, see legend to Chart 1.

Chart 5. A, elution profile of the H1 histone from Kirkman-Robbins hepatoma fractionated on the Amberlite CG-50 chromatographic column (see "Materials and Methods"); Gu-HCl, guanidine hydrochloride; B, electrophoretic patterns of the contents of subsequent chromatographic peaks shown in A; a, initial H1 preparation; b, Peak I; c, Peak II; d, Peak III; e, Peak IV. For electrophoretic conditions, see legend to Chart 1.
values of the molecular weights are summarized in Table 3. The easiest way to prove the basic similarity of H1-slow to, or its diversity from, a typical H1 subfraction could be achieved by comparing the degradation products obtained by the digestion with highly specific or low specific proteases. Following the model experiments of Chapman et al. (9) and Hartman et al. (14), we applied thrombin as the example of highly specific protease.

acetone and could not be removed by dialysis. The same preparative electrophoretic procedure appeared successful for isolation from the mixture of the 3 H1 subfractions present in the chromatographic Peak II.

The amino acid composition of all 4 tumor H1 subfractions and of 2 H1 subfractions from liver is presented in Tables 1 and 2. The approximate molecular weights of the H1 subfractions were evaluated from a calibration curve constructed by plotting the known molecular weights of all 5 main calf thymus histone fractions against their localization on the gel. The

Chart 6. A, elution profile of the H1 histone from normal hamster liver fractionated on an Amberlite CG-50 chromatographic column (see "Materials and Methods"); Gu-HCl, guanidine hydrochloride; B, electrophoretic patterns of the contents of subsequent chromatographic peaks shown in A; a, initial H1 preparation, b, Peak I; c, Peak II; d, Peak III. For electrophoretic conditions, see legend to Chart 1.

Chart 7. An identification scheme for H1 subfractions from hamster hepatoma (A) and normal hamster liver (B). Roman numerals, chromatographic peaks from Amberlite CG-50 columns; Arabic numerals, schematic representation of electrophoretic bands.

Chart 8. Electrophoretic identification of the purified H1-slow and H1-4 subfractions from hamster hepatoma, isolated by preparative chromatography and electrophoresis. The purified H1-4 (d) and H1-slow (e) subfractions were added as internal standards, a, total H1 hamster hepatoma histone with H1-slow added; b, total H1 hamster hepatoma histone with H1-4 added; c, total H1 hamster hepatoma histone. For electrophoretic conditions, see legend to Chart 1.
Table 1
Amino acid composition of the H1 histone subfractions from hamster hepatoma

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>H1-slow</th>
<th>H1-2</th>
<th>H1-3</th>
<th>H1-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.05</td>
<td>2.42</td>
<td>2.09</td>
<td>3.45</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.93</td>
<td>5.79</td>
<td>5.47</td>
<td>5.43</td>
</tr>
<tr>
<td>Serine</td>
<td>10.88</td>
<td>8.56</td>
<td>6.58</td>
<td>7.16</td>
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<tr>
<td>Glutamic acid</td>
<td>5.40</td>
<td>4.61</td>
<td>3.84</td>
<td>4.21</td>
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<tr>
<td>Proline</td>
<td>6.89</td>
<td>8.73</td>
<td>9.05</td>
<td>6.08</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.74</td>
<td>8.10</td>
<td>7.98</td>
<td>8.00</td>
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<tr>
<td>Alanine</td>
<td>18.60</td>
<td>21.59</td>
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<td>24.03</td>
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<td>Half-cystine</td>
<td>11.78</td>
<td>7.60</td>
<td>5.44</td>
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<tr>
<td>Valine</td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Isoleucine</td>
<td>1.04</td>
<td>1.21</td>
<td>0.99</td>
<td>1.04</td>
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<td>Leucine</td>
<td>4.97</td>
<td>4.21</td>
<td>4.34</td>
<td>4.30</td>
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<tr>
<td>Tyrosine</td>
<td>0.51</td>
<td>0.20</td>
<td>0.46</td>
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<tr>
<td>Phenylalanine</td>
<td>1.10</td>
<td>0.89</td>
<td>0.47</td>
<td>0.73</td>
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<tr>
<td>Lysine</td>
<td>23.04</td>
<td>24.64</td>
<td>23.56</td>
<td>23.60</td>
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<tr>
<td>Histidine</td>
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<td></td>
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<tr>
<td>Arginine</td>
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<td>1.45</td>
<td>1.58</td>
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<tr>
<td>Amino sugars</td>
<td>Trace</td>
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Table 2
Amino acid composition of the H1 histone subfractions from hamster liver

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>H1-1</th>
<th>H1-2</th>
</tr>
</thead>
<tbody>
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<td>Cysteic acid</td>
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<td></td>
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<td>Hydroxyproline</td>
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<td></td>
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<td>2.24</td>
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<td>Serine</td>
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<td>6.36</td>
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<tr>
<td>Glutamic acid</td>
<td>4.01</td>
<td>3.74</td>
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<tr>
<td>Proline</td>
<td>9.31</td>
<td>7.29</td>
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<tr>
<td>Glycine</td>
<td>7.75</td>
<td>8.25</td>
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<td>Alanine</td>
<td>26.13</td>
<td>26.07</td>
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<tr>
<td>Half-cystine</td>
<td>6.06</td>
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<tr>
<td>Valine</td>
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<td>4.23</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.02</td>
<td>0.93</td>
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<tr>
<td>Isoleucine</td>
<td>5.42</td>
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<td>Leucine</td>
<td>0.32</td>
<td>0.43</td>
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<tr>
<td>Tyrosine</td>
<td>0.61</td>
<td>0.56</td>
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<tr>
<td>Phenylalanine</td>
<td>23.05</td>
<td>27.22</td>
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<tr>
<td>Lysine</td>
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<td>Histidine</td>
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<tr>
<td>Arginine</td>
<td></td>
<td></td>
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<tr>
<td>Amino sugars</td>
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Table 3
Molecular weights of the H1 histone subfractions from hamster hepatoma and hamster liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>H1 subfractions</th>
<th>Range of the estimated values of M.W.</th>
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</thead>
<tbody>
<tr>
<td>Hamster hepatoma</td>
<td>H1-slow</td>
<td>22,500–23,500</td>
</tr>
<tr>
<td></td>
<td>H1-2</td>
<td>20,000–21,000</td>
</tr>
<tr>
<td></td>
<td>H1-3</td>
<td>20,500–22,000</td>
</tr>
<tr>
<td></td>
<td>H1-4</td>
<td>19,000–18,600</td>
</tr>
<tr>
<td>Hamster liver</td>
<td>H1-1</td>
<td>20,000–21,000</td>
</tr>
<tr>
<td></td>
<td>H1-2</td>
<td>19,500–20,500</td>
</tr>
<tr>
<td></td>
<td>H1-3</td>
<td>20,500–21,000</td>
</tr>
</tbody>
</table>

protease and trypsin for a more advanced comparative degradation. The results obtained by the electrophoresis of the thrombin peptides are shown in Chart 9. The "fingerprints" of the tryptic peptides obtained by digestion of H1-slow and H1-4 from the tumor are compared in Chart 10. Some observations of nonhistone proteins isolated from tumor chromatin revealed...
growing malignant cells only slightly resembling normal hepatocytes. Their high nucleocytoplasmic ratio makes them a convenient source of chromatin components, including histone fractions.

Contrary to all former comparative investigations on normal and neoplastic histone fractions, our experiments revealed, in this particular tumor, the existence of a protein fraction strongly resembling the H1 histones, having a distinctly lower electrophoretic mobility, of H1-slow (2). Therefore, it seems that H1-slow is a separate H1 histone subfraction, characteristic of the investigated tumor cells. To prove this, we should have had larger quantities of purified H1-slow at our disposal. The method of choice for preparative purposes seemed to be the highly selective ion-exchange chromatography on Amberlite CG-50 columns, successfully applied by Sluyser et al. (32) for fractionation of H1 histones from several sources. Unfortunately, the method fell short of our expectations in this particular case. Accidentally, however, in spite of not leading to the isolation of homogeneous subfractions, this procedure highly improved the conditions for their electrophoretic separation just by grouping in one chromatographic peak the slowest and fastest migrating subfractions. The serious increase of the distance between them was crucial for the following preparative electrophoretic separation. In spite of the serious loss caused by the low recovery of the H1 subfractions from gels, we were able to collect sufficient material for further analysis. The decisive proof that the H1-slow is like other H1 histones was obtained by the comparison of the trypsin and thrombin digestion products of H1-slow and other H1 subfractions (Charts 9 and 10). The identity of most of the digestion products proved that both subfractions had a very similar amino acid composition and sequence. Both the amino acid composition (Table 1) and molecular weight values (Table 3) strongly suggested that H1-slow is one of the H1 subfractions. It is longer by a dozen or so amino acids than the others.

In view of what is known about the well-established evolutionary diversity of the H1 histone subfractions in respect to their amino acid composition and sequence, the observed differences may result from several small changes spread along the molecule. Another explanation is that H1-slow differs from other subfractions by possessing an additional chain fragment rich in valine and serine. In order to explain the lower contents of alanine and glycine, one should assume that such an insert is a substitute for a missing one, rich in those amino acids. The stated absence of the sugar residues and the observed diversity of isoelectric points among individual subfractions do not add any valuable information to the knowledge of the nature of H1-slow. How widespread the H1-slow is among normal and neoplastic tissues of hamster and its evolutionary neighbors remains to be investigated.

ACKNOWLEDGMENTS

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REFERENCES


Chart 11. Isoelectric points of the 4 H1 subfractions from hamster hepatoma as determined by electrofocusing in polyacrylamide gel. a, H1-slow; b, H1-2; c, H1-3; d, H1-4.


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