Characterization and chromatin distribution of the H1 histones and high-mobility-group non-histone chromosomal proteins of trout liver and hepatocellular carcinoma

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The H1 histones serve as general repressors of gene expression by inducing the formation of a compact chromatin structure, whereas the high-mobility-group (HMG) non-histone chromosomal proteins have roles in maintaining the structure and function of transcriptionally active chromatin. The distribution of the H1 histone subtypes and HMG proteins among various trout tissues (liver, hepatocellular carcinoma, testis and erythrocyte) was determined. Histone H1b was present in the chromatin of liver, but not in the chromatin of hepatocellular carcinoma, testis or erythrocyte. Nuclease-resistant regions of liver chromatin had elevated levels of histone H1b. Histone H1b was isolated, and the *N*-terminal amino acid sequence of histone H1b was found to be highly similar to that of mammalian histone H1° and duck H5. HMG proteins T1, T2, T3, H6, C, D and F were associated with liver and hepatocellular-carcinoma chromatin, with hepatocellular carcinoma containing higher levels of HMG T1 and F. Testis and erythrocyte had HMG T2 and H6 as their predominant HMG proteins. Most of the HMG H6 of hepatocellular carcinoma, but not of liver, was located in a chromatin fraction that was soluble at physiological ionic strength and enriched in transcriptionally active DNA. These alterations in the chromatin distribution and content of hepatocyte HMG proteins and H1 histone subtypes may contribute to aberrant hepatocyte gene expression in the hepatocellular carcinoma.

INTRODUCTION

The H1 histones are recognized as general repressors of gene activity (Weintraub, 1985; Croston *et al.*, 1991). Repressed chromatin is made inaccessible to the transcription machinery by being assembled into a higher-order condensed chromatin structure, the formation of which is dependent on the action of the H1 histones. The histone H1 class is generally heterogeneous with respect to amino acid sequence (Cole, 1987; Lennox & Cohen, 1988). The proportions of the histone H1 subtypes vary among individual tissues within a particular species, as well as among various species. Further, changes in the relative levels of H1 subtypes have been reported for normal and neoplastic cells (Tan *et al.*, 1982; Davie *et al.*, 1987). Since the histone H1 subtypes differ in their ability to condense DNA and chromatin fragments, it has been proposed that the differential distribution of the H1 subtypes with chromatin domains may generate chromatin regions with different degrees of compaction (Cole, 1987; Lennox & Cohen, 1988).

The high-mobility-group (HMG) non-histone chromosomal proteins are thought to have roles in the structure and function of transcriptionally active gene chromatin. Mammalian and avian chromatin have two major pairs of HMG proteins: HMG 1 and 2, and HMG 14 and 17. HMG proteins 14 and 17 and trout HMG H6 are preferentially associated with transcriptionally active gene nucleosomes, and they are believed to be involved in the maintenance of the DNAase-I-sensitive structure of transcriptionally active genes (Levy W. *et al.*, 1979; Druckmann *et al.*, 1986; Dorbic & Wittig, 1986, 1987; Bustin *et al.*, 1990). Mammalian HMG proteins 1 and 2 and trout HMG T1 and T2, which are associated with linker DNA (the DNA joining the nucleosomes), stimulate transcription, and they function as general transcription factors of RNA polymerase II (Singh & Dixon, 1990).

Rainbow-trout liver and testis chromatin are associated with

HMG T1 and T2, which are similar to HMG 1 and 2, and HMG H6, which is similar to HMG 14 and 17 (Brown *et al.*, 1980; Rabbani *et al.*, 1980; Christensen & Dixon, 1981; Brown & Goodwin, 1983). In addition to these HMG proteins, it has been reported that trout liver chromatin contains HMG proteins C, D and F, which were either absent or present at very low levels in testis (Brown & Goodwin, 1983). However, other investigators did not find these 'liver-specific' HMG proteins (Christensen & Dixon, 1981).

Neoplastic transformation of a cell is thought to be due to the abnormal expression of certain genes. The abnormal expression of these genes may be a result of modification at the gene level and/or changes in chromatin structure. We have been analysing the chromatin structure and composition of trout hepatocellular carcinomas, which consist of basophilic cells with moderately to greatly enlarged hyperchromatic nuclei and high levels of cytoplasmic RNA (Sinnhuber *et al.*, 1977). These tumours were induced by aflatoxin B₁, one of the most potent liver toxins and procarcinogens known, and a naturally occurring food contaminant implicated in the etiology of some human hepatic cancers in Africa and China (Bressac *et al.*, 1991; Hsu *et al.*, 1991). We have demonstrated that trout hepatocellular carcinoma chromatin has decreased levels of the liver histone H1 subtype H1b, but unaltered levels of methylated DNA (Davie *et al.*, 1987). In the present study, we show that histone H1b was non-uniformly distributed in liver chromatin, with micrococcal-nuclease-resistant chromatin regions having the greater levels of H1b. Liver histone H1b was purified, and its *N*-terminal amino acid sequence was found to be highly similar to that of mammalian histone H1° and duck H5. In agreement with the results of Brown & Goodwin (1983), we found that the amounts of HMG proteins C, D and F were considerably higher in liver chromatin than in the chromatins of erythrocyte and testis. We demonstrate that the HMG proteins of liver and hepatocellular-carcinoma chromatin were qualitatively similar but quantitatively

ively different. Further, we show that the chromatin distribution of HMG H6 of hepatocellular carcinoma and liver was markedly distinct.

MATERIALS AND METHODS

Tissues

Livers, hepatocellular carcinomas, erythrocytes and testis were obtained from rainbow trout (*Oncorhynchus mykiss*) at the Oregon State University Food Toxicology and Nutrition Laboratory fish-hatchery facility and at the Rockwood Fish Hatchery, Rockwood, Manitoba. The aflatoxin B₁-induced hepatocellular carcinomas were isolated as described by Davie *et al.* (1987). The tissues were stored at -80°C until use.

Isolation of nuclei

Trout testes, liver and hepatocellular-carcinoma nuclei were isolated as described by Nickel *et al.* (1987), except that $0.5\text{ }\mu\text{g}$ of leupeptin/ml and $1\text{ }\mu\text{g}$ of aprotinin/ml were added to the buffers. Erythrocyte nuclei were isolated by the procedure described for liver.

Fractionation of liver and hepatocellular-carcinoma chromatin

Fractionation protocol 1. Liver nuclei ($0.5\text{--}0.7\text{ mg}$ of DNA/ml) were resuspended in Buffer B [1 M -hexylene glycol/ 10 mM -Pipes (pH 7.0)/ 2 mM -MgCl₂/ 30 mM -sodium butyrate/ 1% (v/v) thiodiglycol/ 1 mM -phenylmethanesulphonyl fluoride/ 1 mM -CaCl₂] (1 g of tissue yields approx. 1.5 mg of DNA). The nuclei were incubated with micrococcal nuclease (50 A_{260} units/ml) for 10 min at 37°C . The digestion was terminated by placing the suspension on ice and adding EGTA to 10 mM . The nuclei were collected by centrifugation (2000 g-min), and the supernatant, which contained linker-region DNA, was discarded. The nuclei were sequentially resuspended and incubated for 20 min in Buffer D [0.2 M -NaCl/ 50 mM -Tris/HCl (pH 7.5)/ 2 mM -MgCl₂/ 25 mM -KCl/ 30 mM -sodium butyrate], followed by 10 mM -Tris/HCl (pH 7.5)/ 1 mM -EDTA. The chromatin, which was solubilized in each solution, was collected by centrifugation, yielding fractions S0.2 and SE. The EDTA-insoluble material was resuspended in 10 mM -Tris/HCl (pH 7.5)/ 1 mM -EDTA, yielding fraction PE. The distribution of DNA among the fractions S0.2, SE and PE was 45.1 ± 4.7 , 24.0 ± 4.2 and $30.9 \pm 5.2\%$ ($n = 3$) respectively. The percentage of DNA in each fraction was determined by the diphenylamine assay of Giles & Myers (1965).

Fractionation protocol 2. Liver and hepatocellular-carcinoma nuclei were resuspended in Buffer N [50 mM -Tris/HCl (pH 7.5)/ 150 mM -KCl/ 5 mM -MgCl₂/ 10 mM -sodium butyrate/ 0.25 M -sucrose/ 1 mM -phenylmethanesulphonyl fluoride containing $0.5\text{ }\mu\text{g}$ of leupeptin/ml and $1\text{ }\mu\text{g}$ of aprotinin/ml] to 1 mg of DNA/ml. The nuclei suspension was made 1 mM in CaCl₂. Nuclei were digested at 50 A_{260} units of micrococcal nuclease/ml at 37°C for 10 min . The reaction was terminated by addition of EGTA to a final concentration of 10 mM and placed on ice. The digested nuclei were collected by centrifugation (2000 g-min), which yielded supernatant 1SF and pellet 1P. The nuclei of 1P were resuspended in 10 mM -Tris/HCl (pH 8.0)/ 1 mM -EDTA and then centrifuged (12000 g-min), yielding supernatant 2SF and pellet 2P.

Isolation and characterization of H1 and HMG protein

Histone H1 and HMG proteins were isolated by making the nuclei suspension in Buffer N or the chromatin fraction 5% in HClO₄ (30 min on ice), and the insoluble material was removed by centrifugation. The acid extracts were dialysed overnight at 4°C against 0.1 M -acetic acid, freeze-dried and redissolved in distilled water. Typically, H1 and HMG proteins were isolated

from 2 ml portions of the nuclear suspension (approx. 1 mg of DNA/ml) or the chromatin fractions. The freeze-dried sample was resuspended in $100\text{ }\mu\text{l}$ of water, except for fractions 1SF and 1SF* (fraction from nuclei incubated without micrococcal nuclease), which were resuspended in $20\text{ }\mu\text{l}$ of water. It should be noted that HMG T proteins are prone to aggregation, which accounts for the variable amounts (e.g. relative to histone H1) of these proteins in the nuclear extracts. However, the relative levels of the HMG T1, T2 and T3 in the various preparations were reproducible.

Liver H1 histones were purified by ion-exchange chromatography on a Bio-Rex 70 column (Seyedin & Cole, 1981). Peptide mapping of the H1 histones was performed as described by Davie (1985). To obtain the peptide maps of histones H1a.1 and H1a.2, these proteins were resolved on acetic acid/ 6.7 M -urea/ 0.375% (w/v) Triton X-100 (AUT) gels, and the gel slices containing these proteins were incubated with the indicated proteinase in the presence of SDS.

PAGE

This was performed as described by Nickel *et al.* (1987). The two systems typically used in this study were 15% -polyacrylamide/AUT and SDS/ 15% -polyacrylamide gels. For high resolution of the H1 histones, 120 mm -long AUT gels were used.

DNA purification and hybridization

The content of DNA sequences in the chromatin fractions was determined by slot-blot analysis (Nickel & Davie, 1989). The ³²P-labelled DNA probes were pPC23, which recognizes the coding region of the protamine genes (Delcuve & Davie, 1987), and pTHC5c, which codes for an abundant mRNA in liver and hepatocellular carcinoma (G. P. Delcuve, J. Sun & J. R. Davie, unpublished work). Enrichment of the transcriptionally active THC5c DNA sequence in chromatin fraction 1SF was calculated by analysing the densitometric scans of autoradiograms from slot-blots. For fraction 1SF, the ratio of THC5c DNA sequences to those in total digested DNA was calculated. To compensate for differences in the sizes of the DNA between fractions, the same ratio was determined for the repressed protamine DNA sequences. The enrichment of THC5c sequences in fraction 1SF was then calculated as the ratio of enrichment of THC5c DNA sequences to enrichment of protamine DNA sequences in that fraction (Nickel & Davie, 1989).

RESULTS

Distribution of trout histone H1 subtypes

H1 histones of trout liver, hepatocellular carcinoma, erythrocyte and testis were separated electrophoretically on long AUT gels, resolving histones H1a and H1b into several forms (Fig. 1). The relative levels of H1a.1 and H1a.2 varied among the trout tissues. H1a.2 constituted 36% , 28% , 32% and 11% of the total H1a population of liver, hepatocellular carcinoma, erythrocyte and testis respectively. Macleod *et al.* (1977) reported that approx. 10% of trout testis histone H1 was not blocked at the N-terminus, suggesting that H1a.2 was the unblocked form of H1a.1. This observation suggests that the efficiency of acetylation at the N-terminus of histone H1a was variable among the tissues, with testis being the most efficient and liver being the least. Testis, but not the other tissues, had several H1 histone species migrating slower than H1a.1 (Fig. 1). These slower-migrating forms corresponded to the phosphorylated forms of histone H1a (Macleod *et al.*, 1977). Histone H1b, which was resolved into three species, and histone 'H5' were located in liver and erythrocyte chromatin respectively (Fig. 1). Histone H1b was not

Fig. 1. Histone H1 subtypes of trout tissues

H1 subtypes isolated from trout liver, hepatocellular carcinoma, erythrocyte and testis were resolved by AUT/PAGE. The gel was stained with Coomassie Blue, and gel patterns were scanned by densitometry. P1, P2, P3 and P4 correspond to the mono-, di-, tri- and tetra-phosphorylated species of H1a.1 respectively.

Fig. 2. Histone H1b is preferentially located in nuclease-resistant regions of liver chromatin

DNA fragments of liver chromatin fractions T (unfractionated chromatin), S0.2, SE and PE were electrophoresed through a 1% agarose gel. The ethidium bromide-stained gel is shown in (a). H1 histones of chromatin fractions S0.2 and PE were resolved by AUT/PAGE. The Coomassie Blue-stained gel was scanned by densitometry (b).

detected in testis, erythrocyte or hepatocellular-carcinoma chromatin. Furthermore, histone H1b was not present in the HClO_4 extracts of five different tumours.

Chromatin distribution of trout liver histone H1b

The distribution of the H1 histone subtypes in chromatin is non-uniform (Huang & Cole, 1984; Jin & Cole, 1986). We have previously described a procedure to fractionate bovine thymus

Fig. 3. Peptide mapping of H1 subtypes

(a) Liver H1 histones (lane d) or H1 subtypes separated on a Bio-Rex column (lane a, H1a; lane b, H1b; lane c, 'H5') were resolved by AUT/PAGE. (b) H1a (lane a), H1b (lane b), 'H5' (lane c) and chicken erythrocyte H5 (lane d) were digested with chymotrypsin (lane 3). The peptides were resolved by SDS/PAGE, and the gel was stained with Coomassie Blue.

and liver chromatin (Davie *et al.*, 1986). The H1 subtypes, including H1^o, were non-uniformly distributed in bovine liver chromatin, with the content of H1^o being elevated in the 0.2 M-NaCl-insoluble nuclease-resistant chromatin fraction. To ascertain the distribution of the histone H1 subtypes in trout liver chromatin, we used the same fractionation procedure (procedure 1 in the Materials and methods section). Fig. 2 shows that the 0.2 M-NaCl-soluble chromatin fragments (fraction S0.2) were shorter than those in fractions SE and PE, with fraction PE containing the longest chromatin fragments. This observation indicated that the chromatin fragments of fractions SE and PE were from more nuclease-resistant chromatin regions than were those of fraction S0.2. Fig. 2 shows that the content of histone H1b was elevated in fraction PE. In chromatin fractions S0.2, SE and PE, H1b represented 8%, 11% and 16% respectively of the H1 histones. This result was reproducible, with the H1 histones of fraction S0.2 having the lowest levels of H1b. In three separate experiments, the level of H1b in the H1 histones of fraction S0.2 was 0.64 ± 0.06 that of the H1b present in the H1 histones of unfractionated chromatin.

Characterization of histone H1b

Trout liver H1 histones were separated by Bio-Rex column chromatography. The chromatographic profile that we obtained

was similar to that reported by Seyedin & Cole (1981), with histone H1a corresponding to chromatographic fractions a-f and histone H1b coinciding with fractions g and h (results not shown). Our preparation of liver H1 histones also contained erythroid histone 'H5', which was eluted after histone H1b. Fig. 3 shows the individual histones resolved on an AUT gel, as well as their peptide maps. The peptide patterns of chymotrypsin-digested trout histones H1a, H1b, 'H5' and chicken erythrocyte H5 were distinct, demonstrating differences in the primary sequence of these four H1 histones. Also, histones H1a.1 and H1a.2 produced the same peptide maps when digested with chymotrypsin or *Staphylococcus aureus* (V8 strain) protease (results not shown).

The *N*-terminal amino acid sequence of trout liver histone H1b was determined. Fig. 4 compares the *N*-terminal sequences of trout H1b, trout H1a, human/mouse H1° and duck H5. Trout histone H1b shared extensive collinear similarity with human/mouse H1° and duck H5. The extent of sequence similarity of trout H1a, human H1° and duck H5 with the H1b sequence (amino acids 4-22) was 42, 53 and 74% respectively. Collinear sequence similarity of this region of trout H1b to that of chicken histone H5 was low (16%).

Tissue-specific distribution of trout HMG proteins

HMG proteins extracted with 5% HClO₄ from the nuclei of trout liver, hepatocellular carcinoma, erythrocyte, and testis were separated on SDS or AUT gels. Since the electrophoretic patterns of liver HMG proteins shown in Fig. 5 were similar to those reported by Brown & Goodwin (1983), the HMG proteins were designated in accordance with the classification of these investigators. Liver contained HMG 14/17-like proteins D, C, H6 and F. G was a putative HMG protein. Through the analysis of one-dimensional and two-dimensional (AUT into SDS) gel

patterns, we determined that HMG proteins C and D had similar electrophoretic mobilities on AUT gels to chicken HMG 14, and HMG F co-migrated with H6 on AUT gels, but ahead of H6 on SDS gels, in agreement with the results of Brown & Goodwin (1983).

The relative levels of the various HMG proteins were similar for liver and hepatocellular carcinoma. However, the abundance of HMG F was higher in the hepatocellular-carcinoma HMG proteins (Fig. 5a, compare HMG F with D or G). This result was consistently observed when the HMG proteins of four hepatocellular carcinoma preparations were analysed (see also Fig. 7). Since liver tissue often possesses nucleated erythrocytes, we were concerned that some of the liver HMG proteins were actually of erythrocyte origin. Fig. 5 shows that the major erythrocyte HMG protein of the HMG 14/17 group is HMG H6. Erythrocyte nuclei had very low levels, if any, of proteins C, D, F and G. Testis also had HMG H6 as its major HMG/17-like protein.

The relative amounts of HMG T proteins also differed among the tissues. Fig. 5(c) shows that perfused liver and hepatocellular carcinoma contained HMG T1, T2 and T3, testis had HMG T1 and T2, and erythrocyte had HMG T2. Further, the relative level of HMG T1 was greater than that of T2 in hepatocellular carcinoma, but the converse was observed for liver. An analysis of the HMG proteins of four hepatocellular-carcinoma preparations demonstrated that the quantity of HMG T1 was either greater than or equal to the amount of T2 (see also Fig. 7).

		5	10	15	20	
Trout H1b	A	E	T	A	A	P
Trout H1a	*	*	*	*	*	*
Human H1°	T	E	N	S	T	S
Duck H5	T	D	S	P	I	P

Fig. 4. Amino acid sequences of the *N*-terminal residues of H1 histones

The *N*-terminal amino acid sequences of trout liver histone H1b, trout testis histone H1a (Macleod *et al.*, 1977), human and mouse histone H1° and duck histone H5 (Alonso *et al.*, 1988) are shown. Parentheses in the trout liver histone H1b sequence indicate that only a tentative identification of these amino acids has been made. Amino acids identical with those in trout liver histone H1b have been given an asterisk, and those amino acids which are different are indicated.

Table 1. Content of liver or hepatocellular-carcinoma nuclear DNA in fraction 1SF or 1SF*

Liver or hepatocellular carcinoma nuclei were incubated in the presence or absence of micrococcal nuclease (50 *A*₂₆₀ units/ml) for 10 min. The percentage of total nuclear DNA in each fraction was determined as described in the Materials and methods section.

Substrate concn. (mg of DNA/ml)	Liver		Hepatocellular carcinoma	
	Nuclear DNA (%)		Nuclear DNA (%)	
	1SF	1SF*	1SF	1SF*
0.93	19.8	1.1	0.93	19.8
0.87	12.6	1.1	0.87	37.5
0.75	21.3	2.7	1.10	22.7
0.77	15.6	1.3	1.02	32.4

Fig. 5. HMG proteins of trout liver, hepatocellular carcinoma, erythrocyte and testis

HMG proteins were isolated from the nuclei of liver (L), hepatocellular carcinoma (H), erythrocyte (E) and testis (T). The proteins were resolved on SDS gels (a), AUT gels (b), or two-dimensional gels (c) where proteins were resolved on a first-dimension AUT gel and then a second-dimension SDS gel. HMG proteins shown in (b), lane L and (c), L, were isolated from nuclei of perfused livers. The AUT gel pattern shown in (b) also contains chicken erythrocyte histones (CE). The gels were stained with Coomassie Blue.

Fig. 6. Distribution of DNA fragment sizes and DNA sequences among liver and hepatocellular-carcinoma chromatin fractions

(a) and (c), DNA fragments isolated from liver and hepatocellular-carcinoma chromatin fractions were electrophoretically resolved on 1%-agarose gels. Lanes a, b and c contained DNA isolated from fractions T (total), 1SF and 2SF respectively. Liver and hepatocellular carcinoma 1SF chromatin fractions contained 19.8 and 22.7% of the nuclear DNA respectively. The gel was stained with ethidium bromide. (b) and (d), DNA of each fraction was denatured and applied to nitrocellulose with a Schleicher and Schuell Manifold II slot-blotter. Filters were hybridized with the indicated ^{32}P -labelled probes, and complementary sequences were quantified by densitometric scanning of the autoradiograms.

Fractionation of hepatocellular-carcinoma and liver chromatin

To fractionate the chromatin of trout liver and hepatocellular carcinoma, we used a procedure which fractionates chromatin into transcriptionally active and repressed regions after selective micrococcal-nuclease digestion (fractionation procedure 2 in the Materials and methods section). Bloom & Anderson (1978) demonstrated that chromatin fragments (fraction 1SF) liberated from micrococcal-nuclease-digested nuclei were enriched in transcriptionally active DNA. To determine the level of endogenous nuclease activity, liver and tumour nuclei were incubated without micrococcal nuclease, yielding fraction 1SF*. Table 1 shows the results of the fractionation of the chromatin from four different livers and four different tumours. Although the nuclei were incubated in a buffer that should decrease endogenous nuclease activity (Vanderbilt *et al.*, 1982), two of the four hepatocellular-carcinoma nuclei preparations had substantial endogenous nuclease activity, liberating approx. 17% of their nuclear DNA into fraction 1SF*. For the tumour nuclei preparations with low endogenous nuclease activities, the amount of nuclear DNA released after micrococcal-nuclease digestion was similar to that liberated from digested liver nuclei (Table 1).

After more extensive chromatin fragmentation, the amount of liver nuclear DNA released into fraction 1SF reached a plateau of approx. 23% (22.7 ± 2.6 ; $n = 6$). The content of tumour nuclear DNA released into fraction 1SF was much higher, reaching 40–50% (based on three separate tumour digestions).

After collection of fraction 1SF, most of the remaining chromatin fragments were solubilized by resuspending the nuclei in a low-ionic-strength buffer containing EDTA, yielding fraction 2SF. Fig. 6 shows that the physiological-ionic-strength-soluble chromatin fragments of fraction 1SF (liver or hepatocellular carcinoma) were shorter than those in fraction 2SF, indicating that the chromatin fragments of fraction 1SF were from nuclease-sensitive open chromosomal regions (Bloom & Anderson, 1978; Koropatnick & Duerksen, 1987). To determine whether fraction 1SF was enriched in transcriptionally active DNA sequences, the relative contents of a transcriptionally active DNA sequence (THC5c) and a repressed DNA sequence (protamine) was determined by slot-blot analysis (Figs. 6b and 6d). The ratios of active/repressed DNA in fraction 1SF [$(\text{THC5c}_{1\text{SF}}/\text{THC5c}_{\text{Total}})/(\text{protamine}_{1\text{SF}}/\text{protamine}_{\text{Total}})]$ of liver and hepatocellular carcinoma were 3.6 and 3.2 respectively. Furthermore, using an anti-ubiquitin antibody in Western-blotting experiments, we observed that ubiquitinated (u) histone H2B was enriched in fractions 1SF of liver and hepatocellular carcinoma (results not shown). We have demonstrated previously that transcriptionally active gene-enriched chromatin fragments were enriched in uH2B, and that the level of uH2B in chromatin was coupled to ongoing transcription (Davie & Murphy, 1990).

Chromatin distribution of liver and hepatocellular-carcinoma HMG proteins

Nuclear DNA of liver or hepatocellular carcinoma was fragmented with micrococcal nuclease, and chromatin fractions 1SF and 1P, which contained all of the nuclear DNA that was not liberated into fraction 1SF, were obtained. Alternatively, nuclei were incubated in the absence of micrococcal nuclease, generating chromatin fractions 1SF* and 1P*. HMG proteins isolated from these chromatin fractions were resolved by SDS/PAGE (Fig. 7). Since the endogenous nuclease activity of liver nuclei was low, the amount of HMG proteins present in liver fraction 1SF* should indicate the extent to which the HMG proteins were extracted from chromatin under the ionic conditions used. Consistent with other reports (Christensen & Dixon, 1981; Kuehl *et al.*, 1980), a small percentage of the HMG proteins T, D, C and H6 was released into fraction 1SF* from mock-digested liver nuclei (Fig. 7, lane L, 1SF*). Approx. 1–5% of the total HMG H6 was located in fraction 1SF*. After micrococcal-nuclease digestion, the level of HMG proteins T2, D, C and F, but not HMG H6, increased in liver chromatin fraction 1SF. Most of the HMG H6 was retained in liver chromatin fraction 1P (approx. 94%). Similar results were obtained with livers that were perfused to remove circulating erythrocytes. To test whether increasing the extent of liver chromatin fragmentation would increase the amount of HMG H6 partitioning into fraction 1SF, liver nuclei were incubated for various times with micrococcal nuclease. Increasing the extent of chromatin fragmentation elevated the amount of HMG proteins D and C, but not H6, in chromatin fraction 1SF (results not shown). Note that the chromatin fragments of fraction 1SF were depleted in histone H1.

After a 10 min incubation, the endogenous nuclease activity of hepatocellular carcinoma nuclei released 17.3% of its nuclear DNA into fraction 1SF*. This level of nuclear DNA release was comparable with that liberated from micrococcal-nuclease-digested liver nuclei into fraction 1SF (19.8%). Similarly to liver fraction 1SF, hepatocellular carcinoma fraction 1SF* acquired

Fig. 7. Distribution of the HMG proteins among the chromatin fractions isolated from liver and hepatocellular-carcinoma nuclei

Liver (L) and hepatocellular-carcinoma (H) nuclei were incubated in the absence or presence of micrococcal nuclease, yielding fractions 1SF* and 1P* or 1SF and 1P respectively, as described in the Materials and methods section. Liver fractions 1SF* and 1SF contained 1.1 and 19.8% of the nuclear DNA, and hepatocellular-carcinoma fractions had 17.3 and 37.5% of the nuclear DNA, respectively. HMG proteins isolated from the chromatin fractions and unfractionated nuclei (T) were electrophoretically resolved on SDS gels, which were subsequently stained with Coomassie Blue. The quantity of HMG proteins loaded on to each lane was extracted from the corresponding amount (in μg) of nuclear DNA present in the fraction (liver: T, 28.1; 1SF*, 0.3; 1SF, 27.8; 1P*, 27.8; 1P, 22.5; hepatocellular carcinoma: T, 26.0; 1SF*, 22.5; 1SF, 49.8; 1P*, 21.5; 1P, 16.3). The last lane, hepatocellular-carcinoma lane H (T), is a nuclear extract from a different tumour.

most of the HMG T1 and T2. However, hepatocellular carcinoma 1SF* and liver 1SF chromatin fractions differed markedly in their content of HMG proteins D, C and H6. Fig. 7 shows that hepatocellular-carcinoma chromatin fraction 1SF* had a greater amount of these HMG proteins than liver fraction 1SF. Approx. 68% of the total hepatocellular-carcinoma HMG H6 was located in fraction 1SF*. The content of HMG proteins D, C and H6 in fraction 1SF from micrococcal-nuclease-digested hepatocellular carcinoma nuclei was slightly greater than that of fraction 1SF*, with approx. 75% of the total HMG H6 being localized in fraction 1SF. This was a representative result for the fractionation of four livers and three hepatocellular carcinoma nuclear DNA preparations.

DISCUSSION

Phosphorylation of the H1 histones has been shown to be confined to proliferating cells, with phosphorylated forms of the H1 subtypes disappearing in the non-dividing cell (Lennox & Cohen, 1988). Trout testis, a tissue engaged in active proliferation, contained phosphorylated forms of H1a, whereas liver and erythrocyte, a non-replicating cell, did not. These results are consistent with the proliferation activities of these tissues. Histone H1a of hepatocellular carcinoma had undetectable levels of the phosphorylated forms of histone H1a, suggesting that the rate of cell proliferation was low in the tumour. Consistent with this observation, an analysis of several different hepatocellular-carcinoma tissue sections demonstrated that mitotic figures were rare and scattered (G. P. Delcuve, J. D. Hendricks, G. Bailey & J. R. Davie, unpublished work). Furthermore, we have measured the level of *c-myc* mRNA, which is strongly correlated with proliferation (Luscher & Eisenman, 1990). Northern-blot analysis of poly-adenylated mRNA from testis, liver and hepatocellular carcinoma demonstrated that testis had a much higher amount of *c-myc* mRNA than either liver or hepatocellular carcinomas, which had low levels of *c-myc* transcripts (G. P. Delcuve & J. R. Davie, unpublished work).

Histone H1b was a component of liver chromatin, but not of the chromatin of erythrocytes, testis or hepatocellular carcinoma. Furthermore, histone H1b was absent from all of the tumours that we have analysed, suggesting that the lack of H1b may be a general feature of hepatocellular-carcinoma chromatin. The amino acid sequence of the *N*-terminus of trout histone H1b was found to be similar to that of mammalian H1 $^{\circ}$ and duck H5. Also, the amino acid composition of trout histone H1b (liver histone H1 fractions g and h; Seyedin & Cole, 1981) was similar to that of other H1 $^{\circ}$ histones (Smith *et al.*, 1984). As with mammalian H1 $^{\circ}$, trout H1b contains methionine, an amino acid not typically found in the H1 histones, has a lower alanine content than trout histone H1a, and has a higher content of arginine than H1a. The ratios of basic to acidic residues of trout histone H1a and H1b were similar, being 6.2 and 6.3 for H1a and H1b respectively (Seyedin & Cole, 1981). These results suggest that trout H1b is a H1 $^{\circ}$ -like histone.

Histone H1 $^{\circ}$ accumulates in tissues with little or no cell division (e.g. adult mammalian liver) and in cells that undergo terminal differentiation. H1 $^{\circ}$ also accumulates in tissue-culture cells when they cease dividing and differentiate. However, for some cultured cancer cells, the level of H1 $^{\circ}$ was not inversely correlated with cell growth rate, with these cancer cells replicating at high rates and having a high H1 $^{\circ}$ content (for example, the hepatoblastoma cell line HepG2; Gabrielli & Tsugita, 1986). Initially, we thought that the absence of trout liver H1 $^{\circ}$ -like histone H1b from hepatocellular-carcinoma chromatin was a consequence of these cancer cells replicating at high rates (Davie *et al.*, 1987). However, our recent results (J. R. Davie & G. P. Delcuve, unpublished work), showing that the hepatocellular-carcinoma cells were not actively proliferating, do not support the idea that the level of histone H1b is inversely correlated with the rate of cell replication.

Roche *et al.* (1985) demonstrated that the level of histone H1 $^{\circ}$ in chromatin correlated with the accessibility of the DNA to micrococcal nuclease, with chromatin with elevated levels of H1 $^{\circ}$ having a greater resistance to nuclease attack. Also, elevating

the content of H1^o enhanced the ability of the chromatin to form a compact configuration with increasing ionic strength. Our results demonstrate that the distribution of histone H1b in liver chromatin was not uniform, with histone H1b being preferentially associated with the nuclease-resistant chromatin regions. It is conceivable that histone H1b may function similarly to histone H1^o in promoting the condensation of the chromatin fibre.

The results of our study clearly demonstrate that the distribution of the trout HMG proteins was tissue-specific. Liver nuclei possessed HMG proteins T1 and T2 of the HMG 1/2 group and proteins D, C, H6 and F of the HMG 14/17 group. In contrast, the major HMG proteins of testis and erythrocyte were T2 and H6. Interestingly, erythrocytes, which have low transcriptional activity and are replication-inactive, had a similar spectrum of HMG proteins to testis, a tissue active in replication. It is conceivable that the higher levels of HMG proteins C, D and F in liver than in erythrocyte or testis reflect the greater diversity of genes expressed in liver than in testis or erythrocyte (Levy W. & Dixon, 1977).

The HMG proteins of hepatocellular carcinoma were similar to those of liver. However, quantitative differences were noted. Hepatocellular carcinoma had a greater quantity of HMG F, a HMG protein that resembles H6 (Brown & Goodwin, 1983). Furthermore, the relative content of HMG T1 was typically greater than T2 in hepatocellular-carcinoma nuclei, whereas in liver nuclei the converse was observed. Thus the level of HMG T1 relative to T2 correlates with the transcriptional activity of the chromatin, with erythrocyte chromatin having the lowest amount of T1 and hepatocellular-carcinoma chromatin possessing the highest content of T1. The distribution of HMG H6 in liver and hepatocellular-carcinoma chromatin also differed. Approx. 70–80% of the hepatocellular-carcinoma HMG H6, but only 6% of liver HMG H6, was located in chromatin fraction 1SF. This chromatin fraction was enriched in transcriptionally active DNA, ubiquitinated histone H2B and HMG proteins, but depleted of H1 histones. It should be noted that these observations do not provide conclusive evidence that HMG H6 and the other HMG proteins were associated with transcriptionally active chromatin. Nevertheless, it is possible that these alterations in the composition (H1 histones and HMG proteins) and organization of hepatocellular-carcinoma chromatin contribute to abnormal hepatocellular gene expression.

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