Coordinate Expression of N-myc 2 and Insulin-like Growth Factor II in Precancerous Altered Hepatic Foci in Woodchuck Hepatitis Virus Carriers

D. Yang, E. Alt, and C. E. Rogler

Marion Bessin Liver Research Center [D. Y. E. A. C. E. R. I.], Department of Pathology [E. A.], and Jack and Pearl Resnick Campus, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

Over 50% of the hepatocellular carcinomas (HCCs) arising in the livers of woodchucks with persistent woodchuck hepatitis virus (WHV) infection contain integrations of WHV DNA within, or immediately adjacent to, a unique and functional N-myc 2 retroposon [G. Fournel et al., Nature (Lond.), 347: 294–298, 1990; Y. Wei et al., J. Virol., 66: 5265–5276, 1992]. The integrations are believed to activate the expression of N-myc 2 by an enhancer insertion mechanism [Y. Wei et al., J. Virol., 66: 5265–5276, 1992]. Since the fetal growth factor insulin-like growth factor II (IGF-II) is also expressed in woodchuck HCCs [X. Fu et al., J. Virol., 62: 3422–3430, 1988; D. Yang and C. E. Rogler, Carcinogenesis (Lond.), 12: 1893–1901, 1991] we sought to determine the earliest stage in hepatocarcinogenesis at which overexpression of N-myc and IGF-II could be detected. The earliest precancerous lesions so far identified in woodchucks are altered hepatic foci (AHFs) [K. Abe et al., Jpn. J. Cancer Res., 79: 466–472, 1988; H. Popper et al., Hepatology (Baltimore), 1: 91–98, 1981]. Using in situ hybridization, we have demonstrated that both the N-myc and IGF-II genes are coordinately overexpressed in nearly all AHFs in precancerous woodchuck livers. In contrast, WHV replication was either repressed or undetectable in the same AHFs. The use of probes selective for N-myc 2 versus N-myc 1 (the normal mammalian homologue) revealed nearly exclusive expression of N-myc 2 in AHFs. Cells within AHFs were generally slow growing, as determined by frequency of histone III-expressing hepatocytes; however, a few fast-growing AHFs, with growth rates nearly equivalent to those of HCCs, were identified. Furthermore, very highly elevated N-myc 2 or IGF-II expression was detected in a few subregions within AHFs which otherwise exhibited a uniformly moderate expression, suggesting that selection for higher levels of N-myc or IGF-II expression may occur within AHFs. These data suggest that coordinate expression of N-myc 2 and IGF-II and repression of WHV replication may be functionally involved in the development of AHFs and that cells expressing very high levels of N-myc and IGF-II may be selectively enriched as AHFs progress to HCC, since high levels of N-myc and IGF-II are common in HCCs.

INTRODUCTION

Soon after the discovery of HBV,1 a link between persistent HBV infection and the occurrence of HCC was observed (1). Since then, several prospective studies have clearly established HBV infection as the primary etiological agent of HCC worldwide (1, 2). In addition to HBV, two other mammalian hepatnaviruses have been identified. These include WHV and ground squirrel hepatitis virus; persistent infection with both of these viruses also has a strong association with HCC (3–7). In the case of woodchucks, precancerous lesions have been identified in the liver which are similar to those occurring in rat liver exposed to carcinogenic regimens (8, 9). The earliest lesions identified in woodchucks are AHFs which are devoid of glucose 6-phosphatase activity and have gained GGT activity (9, 10). From these studies it has been proposed that AHFs are precursors of HCC in woodchucks (9, 10).

The discovery that HCCs arising in the liver of HBV and WHV carriers contained clonally propagated viral DNA integrations (11–15) raised expectations that the viral DNA integrations might lead directly to the identification of protooncogenes associated with hepatocyte transformation. Cloning studies of HBV integrations have led to the identification of human genes which may contribute to hepatocellular transformation (16, 17). However, HBV integration near a known cellular protooncogene has not yet been detected. In contrast, studies of cloned WHV integrations have led to the identification of a unique N-myc gene which is frequently altered by WHV integration (18), as well as integrations of WHV adjacent to the woodchuck c-myc gene (19).

The woodchuck genome contains two N-myc genes, designated N-myc 1 and N-myc 2. Woodchuck N-myc 1 is closely related to other mammalian N-myc genes in sequence and exon structure (18). N-myc 2 is an intronless retroposon which retains N-myc function and can participate with H-ras in tumorigenic transformation of rat embryonic fibroblasts (18). A high frequency of woodchuck HCCs, arising from WHV-infected woodchucks, contain WHV integrations into the N-myc 2 gene. These integrations occur in the 3' untranslated region of the gene, as well as 5' to the gene (18, 20). The N-myc 2 gene is normally silent in adult woodchuck liver; however, the integrations are believed to activate its transcription via an enhancer insertion mechanism (20). Similar findings have previously been described for integrations of murine leukemia virus into the 3' untranslated region of N-myc (21) and avian leukosis virus into the 5' flanking regions of c-myc in chicken lymphomas (22, 23). Interestingly, N-myc 2 is also expressed in some woodchuck HCCs in which no WHV integration has been detected near the N-myc genes (18). Therefore, there may be additional mechanisms for the control of N-myc 2 expression in HCCs which are not dependent on WHV integration.

WHV-associated hepatocarcinogenesis is a multistep process, and N-myc 2 as well as IGF-II, a fetal growth factor (8, 24), are frequently activated in end-stage malignant HCCs in woodchucks. It was of interest to us to study N-myc 2 expression in precancerous lesions in woodchuck liver using in situ hybridization in the hope that such expression data would provide a first step in determining when N-myc 2 may begin to play a functional role in hepatocarcinogenesis. The function of myc genes in promoting either apoptosis or cell growth is regulated by the expression of other genes in several cultured cell types (25–27). Since enhanced expression of IGF-II and down-regulation of WHV have been previously associated with precancerous lesions in woodchucks (8, 24), we also studied their expression in AHFs, to determine whether their expression was altered coordinately with N-myc in the same precancerous lesions. Finally the growth properties of AHFs were analyzed using in situ hybridization with a histone III riboprobe to detect S-phase hepatocytes (8, 28, 29). These approaches have provided a unique view of gene expression in the early stages of hepatocarcinogenesis in WHV carrier woodchucks.
MATERIALS AND METHODS

Identification of Altered Hepatic Foci, Tissue Preparation, and Histological Analysis. The pathologic changes in the livers of 8 woodchucks used in this study are summarized in Table 1. The liver samples were collected from WHV carrier woodchucks at various stages of carcinogenic progression. Samples of precancerous liver and HCCs were divided into three parts. One part was fixed in 4% buffered formaldehyde and embedded in paraffin for histology; a second part was embedded in OCT (Tissue-Tek) and snap-frozen in a dry ice/isopentane bath for in situ hybridization; and the third part was frozen in dry ice/isopentane and stored in liquid N2 for RNA isolation. We identified three animals (woodchucks CW813, 825, and 811) whose livers contained early precancerous lesions with no detectable HCCs and three animals (woodchucks CW819, 824, and 796) whose livers contained precancerous lesions as well as one or more malignant HCCs. Using criteria previously established for precancerous lesions in rats and woodchucks (9, 10, 30–32), we identified liver samples from all 6 animals which contained AHFs. These lesions were composed of basophilic hepatocytes (hematoxylin and eosin staining) and contained GGT-positive hepatocytes (Fig. 2). Altogether, we identified 69 AHFs in liver samples from 6 different woodchucks (Table 1). Most AHFs were associated with portal tracts and involved one to several hepatic lobules. AHFs often encompassed regions of the liver extending completely from portal tract to central vein, and many AHFs contained hepatocytes which extended from both sides of several portal tracts. There was little or no compression of hepatic tissue adjacent to AHFs.

Plasmids and Probe Preparation and in Situ Hybridization. Probe A was a HindIII-Bgl fragment of N-myc 2 (exons 2 and 3) which was subcloned into plasmid pBS (Stratagene) (Fig. 1). Probe B was a SmaI-BamHI woodchuck genomic fragment, and Probe C was a SstI-XbaI woodchuck genomic fragment. These fragments were subcloned into plasmids pBS and pRS, respectively (Stratagene) (Fig. 1). Other cloned complementary DNAs used for in situ hybridization included human IGF-II, histone III, and WHY, as described previously (8). Both 32P-labeled antisense and sense strand riboprobes were transcribed using Sp6, T7, or T3 RNA polymerases as appropriate (Boehringer Mannheim). 32P-labeled DNA probes for Northern blot hybridization were prepared using the random priming method (33). To increase the sensitivity for detecting the N-myc 1-specific transcripts, probes B and C were combined in both in situ hybridization and Northern blot analysis. In situ hybridization was performed using the method of Toth et al. (34) with slight modifications as described previously (8).

Poly A+ RNA Extraction and Northern Blot Analysis. Total RNA was prepared using the guanidine thiocyanate/cesium chloride procedure (35), and poly A+ RNA was prepared using oligodeoxythymidine affinity chromatography (Collaborative Research, Inc., Waltham, MA). Northern blots were hybridized with approximately 80 × 106 cpm of 32P-labeled hybridization probe in 4–5 ml of hybridization buffer as previously described (24). To determine equal loading of poly A+ RNA, the blots were eluted and rehybridized with a 32P-labeled glyceraldehyde-3-phosphate dehydrogenase complementary DNA probe.

Histochemical Staining for γ-Glutamyl Transpeptidase. Six-μm cryostat sections were mounted on gelatin-coated slides, fixed in precooled 95% ethanol for 3 min, and air dried with an electric fan. The γ-glutamyl transpeptidase reaction was performed using the method described by Rutenberg et al. (36), and sections were counterstained with hematoxylin for 2 min, followed by mounting in aqueous mounting medium.

Quantitation of Histone III-positive Hepatocytes. After in situ hybridization with histone H3-2 antisense riboprobes, silver grains over hepatocytes were observed by bright-field microscopy. Ten to twenty fields for each AHF or surrounding liver were picked randomly under X40 magnification, and positive hepatocytes in AHFs and surrounding hepatic tissue were counted separately under ×400 magnification. Color pictures of 15 AHFs, 9 HCCs, and 6 surrounding liver tissues were taken, and all of the parenchymal cells in the pictures were counted to get the average number of parenchymal cells per ×400 field for different tissues. The frequency of S-phase hepatocytes was expressed as a percentage of positive hepatocytes or tumor cells. Student’s t test was performed to compare the frequency of S-phase hepatocytes among different tissues.

Quantitation of N-myc-positive AHFs in the Liver of Woodchuck CW 825. Seven tissue blocks were picked randomly, and cryostat sections were prepared and hybridized with N-myc, IGF-II, and WHY antisense riboprobes. Autoradiography was performed against Kodak X-omat film by exposing the film at −70°C for 3 days. Seventeen N-myc-positive AHFs were identified from the autoradiograms of seven tissue blocks. The sum of the area for each AHF compared to the sum of the areas of all seven sections was calculated, and the data were expressed as a percentage of total area occupied by AHFs.

RESULTS

Expression of N-myc, IGF-II, and WHY in Altered Hepatic Foci. We performed in situ hybridization on serial sections of liver from woodchucks using 32P-labeled sense and antisense riboprobes for both N-myc genes (probes A–C) (Fig. 1), IGF-II, and WHY. In uninfected woodchuck livers of animals CW806 and CW873, no detectable N-myc, IGF-II, or WHY hybridization was observed. However, N-myc expression was detected in 65 of 69 AHFs (94%) identified in liver specimens from WHV carrier woodchucks (Table 1). Autoradiograms of serial sections from two liver specimens which
were hybridized with N-myc, IGF-II, and WHV antisense riboprobes, respectively, as illustrated in Fig. 2A. The dark regions of the autoradiograms represent areas of positive riboprobe hybridization. The AHFs in these sections which were N-myc-positive were also IGF-II-positive. However, the same N-myc- and IGF-II-positive lesions were either nonpermissive for WHV expression (Fig. 2A, light areas in the sections) or “semipermissive” (Fig. 2A, speckled appearance). These data illustrate coordinate overexpression of N-myc and IGF-II and down-regulation of WHV in woodchuck AHFs on the tissue level. The absolute level of N-myc expression varied considerably among different AHFs, as judged by the intensity of autoradiogram exposure (Fig. 2A) and dark-field microscopy (Fig. 2B). Some AHFs contained only slightly elevated steady-state N-myc RNA levels, whereas others contained levels equivalent to the high levels routinely observed in woodchuck HCCs.

Microscopic analysis, under bright- and dark-field illumination, of a complete set of serial sections from one typical AHF (marked by an arrow in Fig. 2A) is shown in Fig. 2B. Bright-field illumination of a hematoxylin and eosin-stained cryostat section illustrates the more basophilic nature of the cells in the AHF, and these cells have a slightly larger nuclear:cytoplasmic ratio (Fig. 2B1). Fig. 2B2 (bright-field microscopy) illustrates a typical heterogenous pattern of GGT expression in this AHF, in which the GGT-positive cells extended outward from a portal tract in the interior of the AHF and peripheral hepatocytes in the AHF were GGT-negative.

In situ hybridization of the N-myc antisense riboprobe (probe A) revealed a strikingly uniform expression throughout the entire AHF (Fig. 2B3), which was typical of most AHFs which expressed N-myc. In situ hybridization using an IGF-II antisense riboprobe revealed elevated expression in exactly the same pattern as N-myc (Fig. 2B4). However, in the case of IGF-II, a basically uniform expression pattern throughout the AHF was mixed with areas of highly elevated IGF-II expression. The areas of highly elevated IGF-II expression were predominantly localized around the vessels, at the periphery of the AHF (Fig. 2B4, curved arrow). Hybridization of the WHV antisense riboprobe (Fig. 2B5) revealed a heterogeneous pattern of WHV expression in which cells at the periphery of the AHF were completely negative for WHV RNA while the cells in the interior were semipermissive. The WHV expression pattern roughly correlated with that of GGT expression in AHFs. This is evident by comparing the bright-field GGT staining pattern with the dark-field hybridization pattern (bright spots) in Fig. 2, B2 and B5.

Hybridization of the histone H-3.2 antisense riboprobe detected scattered histone III-positive cells throughout the AHF and the surrounding tissue, as shown in Fig. 2B6. Quantitation under bright-field illumination, which allowed the identification of the histone III-positive cells as hepatocytes, nonparenchymal cells such as Ito cells, or mononuclear cells (data not shown), revealed that 1% of hepatocytes were positive for histone III mRNA in this AHF. This level is slightly elevated compared to surrounding liver, in which the average frequency was less than 0.3%. Interestingly, the very small region, marked by a curved arrow in Fig. 2B4, which corresponded to a region of highly elevated IGF-II expression, had a frequency of 2.5% histone III-positive (S-phase) hepatocytes. High-magnification analysis of the silver grains localized over various cell types revealed that expression of N-myc, IGF-II, WHV, and histone III occurred in hepatocytes (data not shown). Hepatocytes identified in the sections were also positive for albumin expression as judged by in situ hybridization (data not shown).

Our survey also showed that four AHFs contained subregions or “foci within foci,” in which N-myc expression was highly elevated in comparison to a moderate base level of expression in the remainder of the AHF. In the AHF illustrated in Fig. 3, one group of hepatocytes contained a very high level of N-myc RNA on a background of moderately elevated N-myc expression (Fig. 3B). Bright-field microscopy of cells in the area containing highly elevated N-myc RNA revealed that they were densely packed small hepatocytes, with a higher nuclear:cytoplasmic ratio than that of surrounding hepatocytes (Fig. 3C, curved arrow). WHV expression was completely negative in a serial section of the exact same region (Fig. 3D), suggesting that selection of cells which are highly N-myc-positive and WHV-negative may occur within subgroups of cells in AHFs. Cells in this same region expressed IGF-II at a moderate level (data not shown). Three AHFs also contained subregions with high IGF-II expression, as shown in Fig. 2B4. These high IGF-II-expressing “foci within foci” also exhibited a higher nuclear:cytoplasmic ratio and were nonpermissive for WHV replication (data not shown). However, we failed to identify any “foci within foci” which contained highly elevated expression for both IGF-II and N-myc.

The N-myc 2 Allele Was Selectively Overexpressed in Altered Hepatic Foci of Woodchucks. We next determined which N-myc gene, N-myc 1 or N-myc 2, was overexpressed in woodchuck liver containing AHFs. Poly A+ RNA was isolated from two woodchuck livers (CW813 and CW825), which contained multiple AHFs but no detectable HCC, and from one uninfected woodchuck liver (CW806). Hybridization of Northern blots with probe A which detects both N-myc 1 and N-myc 2 transcripts revealed a single 2.3-kilobase RNA, which is the size predicted for mRNA transcribed from the unarranged N-myc 2 retrotransposon (Fig. 4). No 2.9-kilobase RNAs, the size expected for N-myc 1 mRNAs (18), were observed. The blot was eluted and rehybridized with Probes B and C which are specific for N-myc 1 and these probes did not hybridize to the blot, whereas they did hybridize to testes RNA (data not shown).

It was possible that N-myc 1 could be expressed in a few AHFs and that rare N-myc 1 transcripts would not be detected by Northern blot hybridization. Therefore, additional sections of woodchuck liver were hybridized with either riboprobe A or riboprobes B plus C. Hybridization with riboprobe A detected N-myc expression in AHFs which had been previously identified in the liver sections, while hybridization of serial sections with a combination of probes B and C revealed

### Table 1: Summary of pathological changes and in situ hybridization results for WHV carrier woodchuck livers

<table>
<thead>
<tr>
<th>L.D. of woodchuck</th>
<th>WHV infection</th>
<th>Age (years)</th>
<th>Pathological changes</th>
<th>No. of AHF examined</th>
<th>Relative level of N-myc 2 expression</th>
<th>Expression of N-myc 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW811</td>
<td>+</td>
<td>~4</td>
<td>CAH AHF HCC</td>
<td>1</td>
<td>0 1 0 0 0 0</td>
<td>(+)</td>
</tr>
<tr>
<td>CW813</td>
<td>+</td>
<td>~3</td>
<td>- Multiple -</td>
<td>14</td>
<td>2 7 4 1 14 0</td>
<td>(++)</td>
</tr>
<tr>
<td>CW825</td>
<td>+</td>
<td>~3</td>
<td>- Multiple +</td>
<td>31</td>
<td>2 17 8 4 30 1</td>
<td>(++)</td>
</tr>
<tr>
<td>CW796</td>
<td>+</td>
<td>~4</td>
<td>- Multiple -</td>
<td>12</td>
<td>0 7 3 2 11 1</td>
<td>(++)</td>
</tr>
<tr>
<td>CW819</td>
<td>+</td>
<td>~5</td>
<td>- Multiple +</td>
<td>7</td>
<td>0 3 4 0 7 0</td>
<td>(++)</td>
</tr>
<tr>
<td>CW824</td>
<td>+</td>
<td>~5</td>
<td>- Multiple -</td>
<td>4</td>
<td>0 3 1 0 4 0</td>
<td>(++)</td>
</tr>
<tr>
<td>CW806</td>
<td>+</td>
<td>~4</td>
<td>- Multiple -</td>
<td>0</td>
<td>0 0 0 0 0 0</td>
<td>(++)</td>
</tr>
<tr>
<td>CW873</td>
<td>+</td>
<td>~4</td>
<td>- Multiple -</td>
<td>0</td>
<td>0 0 0 0 0 0</td>
<td>(++)</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>~4</td>
<td>- Multiple -</td>
<td>69</td>
<td>4 38 20 7 67 2</td>
<td>(++)</td>
</tr>
</tbody>
</table>

---

a CPH, chronic persistant hepatitis; CAH, chronic active hepatitis; AHF, altered hepatic foci.
b +, no expression detectable; +, low-level expression; +++, medium-level expression, ++++, high-level expression.
Fig. 2. A, N-myc, IGF-II, and WHV expression in persistently infected woodchuck livers. Autoradiograms of serial sections of two tissue blocks hybridized with WHV, N-myc, or IGF-II antisense riboprobes. Areas of positive hybridization appear as dark regions of the sections. B, Microscopic analysis of gene expression in serial sections of a single AHF. The AHF analyzed is the one marked by arrows in A. In all of the serial sections long solid arrows denote the outer boundaries of the AHF, open arrows denote portal tracts, solid arrowheads denote central veins, and curved solid arrows denote specialized structures. The pattern of portal tracts and central veins provides landmarks to facilitate direct comparison of the serial sections. 1, structural organization of the AHF and surrounding liver viewed by bright field microscopy. H&E, ×40. 2, variable GGT expression within the AHF. Bright-field microscopy, hematoxylin counter-staining, ×40. 3-6, dark-field micrographs in which silver grains appear as bright spots, ×40. 3, N-myc expression. 4, IGF-II expression. Curved arrow, highly elevated expression of IGF-II in the subregion of the AHF. 5, WHV expression detected with a WHV antisense riboprobe. Note the low and variable expression, which is strongest in GGT-positive regions (compare to 2). 6, histone H3–2 expression. Note higher frequency of histone III-positive cells in the foci compared to the surrounding liver tissue.
N-myc 2 AND IGF-II EXPRESSION IN PRECANCEROUS LESIONS

Fig. 3. Identification of focal regions of high N-myc expression within the context of an AHF with moderate N-myc expression. Open arrows, portal tract; solid arrows, edge of AHF; curved arrows, specific gene expression in the same subregion of the AHF in serial sections. A, bright-field microscopy illustrating variable GGT expression in the AHF, × 40. B, N-myc expression in a serial section of A viewed by dark-field microscopy, × 40. Curved arrow, focal region of higher N-myc expression. Note moderate level of N-myc expression throughout the AHF. C, bright-field microscopy of the region containing high N-myc 2-expressing cells identified in B. Curved arrows, junctional border between the two different levels of N-myc expression, high level to the right and moderate level to the left. H&E, × 400. D, WHV expression detected by antisense riboprobe. Curved arrow, edge of the region with reduced WHV expression. Dark-field microscopy, × 100. This is the same area marked with curved arrows in A, B, and C.

a faint positive hybridization (above the level of sense strand negative control) in only 2 of 69 AHFs (data not shown). Therefore, N-myc 1 may be expressed in a few AHFs; however, N-myc 2 is the highly predominant N-myc allele expressed.

N-myc 2 Allele Was Predominantly Overexpressed in Woodchuck HCC Tumors. Thirteen HCCs from woodchucks CW796, CW824, CW819 and three additional animals were hybridized with N-myc probe A or probes B plus C. All 13 tumors were highly positive for probe A but negative for probes B and C (data not shown), suggesting that N-myc 2 was predominantly expressed in these HCC tumors. This is a higher rate of N-myc expression than has been previously reported in woodchuck HCCs (18, 20). Furthermore, these tumors also contained elevated IGF-II expression, as we previously reported (8).

Highly Variable Growth Properties of N-myc 2-positive AHFs. The expression of histone III gene has been shown to be cell cycle regulated and tightly coupled with de novo DNA synthesis (S phase) (28, 29). We have previously reported the application of histone H3.2 in situ hybridization to analyze the relative growth properties of woodchuck HCCs (8). A control experiment, in which a partial (two-thirds) hepatectomy was performed on mice, demonstrated that the frequency of S-phase hepatocytes, as determined by histone H3.2 in situ hybridization, was directly related to the frequency of [3H]thymidine-labeled hepatocytes, demonstrating that histone III expression is a reliable approach to analyzing liver proliferation (data not shown). Therefore used this approach to estimate the relative growth rate of hepatocytes in AHFs versus surrounding liver. Hepatocytes in S phase were very rare in liver tissue surrounding AHFs (0.22%); however, analysis of 46 AHFs revealed an average 2-fold increase, to 0.45%, of S-phase hepatocytes (Fig. 5A ). This value was 7 times lower than the average frequency of S-phase tumor cells in woodchuck HCCs (3.3%). A histogram displaying the frequency of S-phase cells in AHFs showed that, while the majority of AHFs had a mitotic index that was the same or only slightly elevated compared to surrounding liver, a subset of AHFs had very high mitotic indexes. Two AHFs had mitotic indexes of 1.25–2%, and two others had mitotic indexes of 2–2.7%. AHFs with the highest mitotic indexes corresponded to the foci which expressed N-myc 2 and IGF-II at moderate levels.

DISCUSSION

By combining a classical approach (i.e., describing AHFs according to their basophilic staining properties and γ-glutamyl transpeptidase activity) with in situ hybridization for a protooncogene (N-myc), a fetal growth factor (IGF-II), and WHV replication, we have obtained a unique view of precancerous lesions in woodchuck liver. AHFs described in the present study are the earliest precancerous lesion characterized in woodchucks (9), and they closely resemble early...
altered hepatic foci observed in rats exposed to carcinogenic regimens (37, 38). In rats, most AHFs eventually disappear (remodeling), and only a few progress to become persistent lesions (37, 38). If replicative activity is a measure of whether an AHF will persist or not, it is clear that the vast majority of AHFs in woodchucks will not persist. Our observations that most AHFs express both N-myc and IGF-II and that this phenotype is also common in woodchuck HCCs suggest that overexpression of both N-myc and IGF-II has important functional significance in WHV-induced hepatocellular carcinogenesis. However, since only a few AHFs (two) had mitotic indexes approaching that of HCCs, the moderately elevated expression of these two genes alone is not sufficient to increase the mitotic activity of hepatocytes in AHFs to that of fully malignant HCCs. Subsequent (epi)genetic event(s) may be required to establish the growth properties of malignancy.

Our in situ hybridization data show that a distinction can be made between moderate levels of N-myc or IGF-II expression generally observed in AHFs and subregions within AHFs which exhibit highly elevated expression of these genes. Several cases were observed in which small groups of cells within AHFs (foci within foci) exhibited either highly elevated N-myc 2 or IGF-II expression and a coordinate loss of WHV transcription and replication. Cells with this pattern of gene expression may be at a selective advantage for tumor progression, since a major group of fully malignant HCCs in woodchucks frequently express high levels of N-myc and IGF-II; they are also nonpermissive for WHV replication (8, 18, 20, 24). It should be pointed out that another class of HCCs exists in woodchucks in which IGF-II is not expressed; these tumors are permissive for WHV replication (24).

In an effort to estimate the number of AHFs per liver which might progress to HCC, we made calculations of possible selection events needed for progression. To estimate the number of AHFs in an entire woodchuck liver, we selected 7 random liver sections from woodchuck liver CW825 and calculated the percentage of their area which was occupied by AHFs. Most of the AHFs were spherical, and our calculations (see "Materials and Methods") estimated their area to be 3.5% of the total area of the sections. We calculated that the average volume of each AHF was approximately 1.5 mm³ (assuming a random distribution in our 7 sections and a spherical 3-dimensional structure).

Fig. 4. Northern blot analysis of N-myc expression in woodchuck liver and HCC. Lanes 1 and 2, 12 μg poly A⁺ RNA from precancerous livers containing AHFs from woodchucks CW825 and CW813, respectively. Lane 3, 12 μg poly A⁺ RNA from an uninfected woodchuck liver (woodchuck CW806). Lane 4, 1 μg poly A⁺ RNA from a woodchuck HCC (woodchuck CW824 tumor 1). The blot was hybridized with 32P-labeled N-myc probe A. Note the 2.3-kilobase RNA, of the size predicted for normal N-myc 2 mRNA in Lanes 1 and 2. Bottom, hybridization with a glyceraldehyde-3-phosphate dehydrogenase probe, illustrating equal loading of poly A⁺ RNAs in Lanes 1–3.

Fig. 5. Quantitation of S-phase hepatocytes in AHFs. A, frequency of S-phase hepatocytes in: liver surrounding precancerous lesions (L), 20 sections from 5 livers analyzed; altered hepatic foci (AHF), 46 AHFs analyzed; and hepatocellular carcinomas (HCC), 9 HCC analyzed. **, value significantly different from that of L or AHF samples (P < 0.001, Student's t test). B, bar graph illustrating the frequency of AHFs with different mitotic indices. Normal woodchuck liver falls in the 0–0.499% categories.
Since the total liver weight was 75.0 g (volume of approximately 7.5 \( \times 10^4 \) mm\(^3\)), we estimated that the entire liver contained approximately 1750 AHFs. Since 94% of the AHFs expressed N-myc 2, the entire liver would contain approximately 1645 N-myc 2-positive AHFs. In our survey of 69 AHFs, 4 (approximately 6%) contained subregions (foci) of highly elevated N-myc expression equivalent to that found in HCCs. This would correspond to 99 foci in the entire liver with highly elevated N-myc 2 expression.

Since the number of HCCs which develop in woodchucks is almost always in the range of one to 10 (rarely above 5 but sometimes up to 14), it is clear that additional epigenetic and/or genetic factors are required for N-myc 2-positive foci to develop into fully malignant HCCs. The identity of these factors is currently unknown. For the sake of argument, we supposed that one such factor could be highly elevated expression of IGF-II. In this and previous surveys of IGF-II expression in precancerous lesions, we have demonstrated a pattern of moderate and highly elevated IGF-II expression in AHFs, similar to that of N-myc 2. In the current survey, 5% of the AHFs contained subregions of highly elevated IGF-II, equivalent to that found in HCCs. However, we have not identified any AHFs which contain highly elevated transcripts of both IGF-II and N-myc in the exact same cells. This event might be expected to occur only rarely. Therefore, if 5% of the highly N-myc-positive foci develop subregions of highly IGF-II-positive cells, as estimated from our survey, this would bring the number of lesions highly positive for both IGF-II and N-myc 2 to 4 or 5. This is in the range of the number of HCCs which often develop in woodchucks.

Myc proteins interact with other cellular proteins in the control of differentiation and/or cellular proliferation. The interaction of c-myc with the transcription factor Max, or its murine homologue Myn, can either enhance or reduce the ability of myc to cooperate with Ras in transformation assays, depending on the relative levels of the two proteins in cells (39–42). In addition, myc proteins interact with specific forms of the retinoblastoma tumor suppressor protein, but the consequences of these interactions in cellular transformation are not understood (43). C-myc also induces apoptosis in a wide variety of cell types, and coexpression of the Bcl-2 protooncogene specifically abrogates c-myc-induced apoptosis without affecting c-myc mitogenic functions (26, 27). A "two signal" model is emerging in which c-myc can provide the first signal, leading to apoptosis or to progression into the cell cycle, and other gene products, such as Bcl-2 or IL-3 (25), may provide a second signal to inhibit apoptosis and allow the mitogenic actions of c-myc to prevail (26).

The extent to which the observations for c-myc apply to N-myc 2 in woodchuck AHFs is unknown. However, activation of c-Myc has also been reported in woodchuck HCCs (19). Furthermore, woodchuck HCCs which do not express N-myc express c-myc (44). Therefore, N-myc and c-myc may substitute for each other, at least in woodchuck HCCs. In any event, it is most likely that other gene products dictate the functional outcome of N-myc 2 expression in hepatocytes. The coordinate expression of IGF-II in AHFs is consistent with a possible role in regulating N-myc function, perhaps by interfering with the apoptosis pathway as IL-3 does in myeloid cells (25).

The mechanism for coordinate regulation of N-myc 2 and IGF-II expression in AHFs is unknown. Persistently infected livers from hepadnavirus carriers undergo a fluctuating cycle involving cell death and regeneration, accompanied by an inflammatory responses. Nearly all of the AHFs we identified were associated with a portal tract inflammatory cell lesions capable of producing a wide variety of cytokines. In this regard, the regulatory sequences upstream of the woodchuck N-myc 2 gene contain a tandem repeat of the IL-6 consensus response element, which may render N-myc 2 inducible by this cytokine (18, 45), since IL-6 receptors are present in liver (45). Furthermore, tumor necrosis factor \( \alpha \) has recently been shown to repress hepatitis B virus surface antigen expression in transgenic mouse hepatocytes when administered systemically at physiological concentrations (46). Thus, localized production of this cytokine in the liver may contribute to the reduction of viral transcription and replication in AHFs. The possible role of other cytokines in controlling the balance between apoptosis and hepatocyte regeneration in WHV infected liver is an important area for further study.

ACKNOWLEDGMENTS

The authors would like to thank Genevieve Fourel and Marie-Annick Buen- dia for kindly providing the woodchuck N-myc 1 and N-myc 2 clones used in this study. We also thank David A. Shafritz for his critical evaluation of the manuscript and advice and encouragement during the course of the study, and E. Bobe for typing the manuscript.

REFERENCES

20. Wei, Y., Fourel, G., Ponzoett, A., Silvestro, M., Toliolais, P., and Buendia, M. A.


Coordinate Expression of N-myc 2 and Insulin-like Growth Factor II in Precancerous Altered Hepatic Foci in Woodchuck Hepatitis Virus Carriers

D. Yang, E. Alt and C. E. Rogler


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/9/2020

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.