Expression of Retroviral Sequences and Oncogenes in Rat Liver Tumors Induced by Diethylnitrosamine


ABSTRACT

The expression of three cellular oncogenes (c-myc, c-Ha-ras, and c-erb-raf), the epidermal growth factor receptor gene, and two endogenous retrovirus-like sequences [rat leukemia virus (RaLV) and 30S] was examined in control rat livers and in 16 liver tumors. The tumors were induced in Sprague-Dawley male and female rats by a single i.p. injection of diethylnitrosamine at 1 or 2 days after birth, followed by dietary exposure to phenobarbital beginning at weaning. Increased expression of c-myc was seen in most of the tumors, but there was no consistent increase or decrease in expression of c-Ha-ras or c-erb-raf. It is of interest that a number of the tumor samples showed a decrease in epidermal growth factor receptor RNA. In all of the tumors, including both hepatocellular adenomas and carcinomas, there was a marked increase in expression of the endogenous RaLV sequence, and over 90% of the tumors displayed increased expression of the 30S endogenous retroviral-like sequence. There was a very low level of expression of the RaLV and 30S sequences found in the control livers. The extent of expression of the RaLV and 30S sequences in individual tumors did not correlate with the extent of expression of c-myc or c-Ha-ras. Although increased expression of certain endogenous retrovirus-related sequences appears to be a common finding during rat liver carcinogenesis, the significance of this finding remains to be determined.

INTRODUCTION

All rats normally contain in their genome about 80 copies of RaLV2-related DNA sequences, as well as 20–40 copies of an endogenous replication-defective retrovirus-like sequence designated 30S (1, 2). In previous studies from this laboratory, enhanced expression of endogenous Moloney murine leukemia virus-like and intracisternal A particle-related sequences has been found in both carcinogen-induced and spontaneous mouse liver tumor (3). We have also observed increased expression of these endogenous murine retrovirus-like sequences in carcinogen-induced murine skin tumors (4) and in carcinogen or radiation-transformed murine fibroblast cell cultures (5). The ubiquity of endogenous retrovirus-like sequences in mammalian species, their similarity to transposable elements in lower organisms, and their frequent expression in tumors have raised several hypotheses regarding their roles in normal development and in cancer (6). Altered expression of cellular oncogenes (or protooncogenes) is also common in tumor tissues and in transformed cell cultures (7, 8), although the full significance of these changes with respect to the process of neoplastic transformation is not known. The most frequently investigated oncogenes belong to the ras and myc gene families. Parallel studies on altered expression of these (and other) cellular oncogenes and altered expression of endogenous retrovirus-related sequences have not, however, been done during multistage carcinogenesis in rat liver. Hence, the present studies were designed to examine the expression of both cellular oncogenes and endogenous retrovirus-like sequences during the development of carcinogen-induced rat liver tumors.

MATERIALS AND METHODS

Animals and Tissue Samples. As described previously (9), liver tumors were initiated neonatally in Sprague-Dawley rats (pregnant females obtained from Charles River Laboratories, Wilmington, MA) by a single i.p. injection of DEN. At weaning, these rats were placed on a diet containing phenobarbital to promote tumor expression. Normal Sprague-Dawley rats (200 days old) were ordered from Harlan Sprague-Dawley, Indianapolis, IN. Animals were sacrificed by cervical dislocation; livers and hepatic tumors were quickly removed, frozen in liquid nitrogen, and stored at −70°C. Details regarding the history and pathological diagnosis of each tissue sample are provided in Table 1.

RNA Preparation and Northern Blot Analysis. Frozen tumors and livers were then homogenized in guanidine monothiocyanate using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Total RNA was isolated by the method of Karin et al. (10). The polyadenylated RNA fraction was isolated by passage of this RNA through oligodeoxythymidylylrate cellulose columns (Collaborative Research, Waltham, MA) (11). Four-μg samples of polyadenylated RNA were analyzed by electrophoresis on 1% agarose gels containing 6% formaldehyde and transferred to Hybond-N hybridization transfer membranes (Amersham Corporation, Arlington Heights, IL). The membranes were then irradiated with UV for 2–5 min. Hybridization to appropriate 32P-labeled probes (see below) and autoradiography were performed according to Wahl et al. (12). After hybridization to one probe and autoradiography, some filters were washed extensively and rehybridized to a second probe. A nonpolyadenylated RNA sample was included in each gel to provide molecular size markers (28 and 18S). In order to visualize the markers and the amount of RNA present in each lane, the gels were stained with ethidium bromide. The ethidium bromide staining indicated that all lanes contained approximately equivalent amounts of RNA. The relative abundance of specific transcripts in the different lanes was determined by densitometric analysis of the autoradiographs.

Hybridization Probes. The following DNA inserts were used: 30S, 5.4-kilobase SacI fragment excised from a pBR322 recombinant (13); RaLV, 8.2-kilobase SacI fragment excised from the vector AgtWES-AB (14); Ha-ras-specific insert, 460-base EcoRI fragment excised from the BS-9 clone (15); v-myc, 1.5-kilobase PstI fragment excised from the MCE38 insert subcloned in pBR322 (16); EGF receptor 64-3, 768-base EcoRI fragment excised from the clone pUC12 (17); c-erb-raf, 1617-base EcoRI fragment excised from the clone pUC19 which was obtained from Dr. U. R. Rapp. Purified fragments were 32P labeled by nick translation (18).

RESULTS

Expression of Endogenous Retroviral Sequences

RaLV. Northern blot hybridization analysis was used to quantitate the expression levels of retrovirus-related sequences in rat livers. Messenger RNA transcripts homologous to RaLV were absent or barely detected in the normal livers of 209-day-

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2 The abbreviations used are: RaLV, rat leukemia virus; DEN, diethylnitrosamine; EGF, epidermal growth factor; kb, kilobase.
Table 1  Treatment regimens and pathological findings in Sprague-Dawley rats administered diethylnitrosamine and phenobarbital to induce liver tumors
For additional details see "Materials and Methods" and Ref. 9.

<table>
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<th>Animal</th>
<th>Sex</th>
<th>Dose (μg)</th>
<th>Age (days)</th>
<th>Phenobarbital (% in diet)</th>
<th>Age at sacrifice (days)</th>
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</table>

old male or female rats. These transcripts were, however, present at rather high levels in all of the 12 hepatocellular adenomas and in all of the 4 carcinomas examined (Fig. 1). The most prominent transcript was about 6.8 kb. An 8.0-kb transcript was very prominent in the liver tumor sample 17, and a low level of this transcript was also seen in liver tumors 20 and 21. Other RNA species of about 4.5 and 1.5 kb homologous to the RaLV probe were also detected in all of the 16 liver tumors when the autoradiograms were exposed for about 4 days.

**30S.** High levels of mRNAs homologous to the 30S retrovirus-related probe were also seen in all of the 4 hepatocellular carcinomas and in 11 of the 12 hepatocellular adenomas (Fig. 1). The major transcript was about 8.4 kb. In addition, a minor 5.6-kb transcript was also observed in the tumor samples. When the autoradiograms were exposed for a longer period of time both of these transcripts were barely detected in the three normal female livers and were seen at a low level in the three normal male livers (Fig. 1).

**Expression of Cellular Oncogenes and the EGF Receptor Gene**

**c-myc.** All of the 4 hepatocellular carcinomas and 9 of the 12 adenomas displayed detectable levels of a 2.5-kilobase c-myc transcript (Fig. 2A). The levels of this transcript varied considerably between individual tumors. This transcript was not detected, however, in any of the normal liver samples.

**c-Ha-raz.** H-ras related transcripts, about 1.4 kb in size, were observed in all of the normal livers and liver tumor samples (Fig. 2A). The relative abundance of these transcripts was not significantly different between the normal liver and tumor samples when quantitated by densitometric analysis of the autoradiographs.

**EGF Receptor.** EGF receptor-related transcripts that were 10.5, 7.5, and 5.8 kb in size were found in all of the normal liver and tumor samples (Fig. 2B). The levels of expression of
these transcripts were markedly reduced in 7 of the 12 adenomas and all 4 of the hepatocellular carcinoma samples when compared to the normal liver samples. This decrease was confirmed by densitometry (data not shown here).

c-3-raf. RNA samples homologous to a c-3-raf probe were observed in all of the normal liver and tumor samples (Fig. 2B). The major transcript was about 2.3 kb. In addition, a minor 4.0 transcript was detected in all of the normal liver and tumor samples. Three of the 4 hepatocellular carcinomas and 6 of the 12 hepatocellular adenomas displayed a significant decrease in the levels of these transcripts when compared to the normal liver samples.

DISCUSSION

The present studies demonstrate a marked increase in expression of endogenous retroviral sequences related to RaLV and 30S proviral DNA sequences in DEN-induced rat hepatocellular adenomas and carcinomas, when compared to age-matched normal livers. These results indicate that there is altered expression of specific endogenous retroviral sequences in carcinogen-induced rat liver tumors. The pattern of expression of these sequences were in general similar in the hepatocellular adenomas and carcinomas. Previous studies from this laboratory have demonstrated that there is also increased expression of endogenous retrovirus-related DNA sequences in carcinogen-induced rat liver tumors. The pattern of expression of these sequences were in general similar in the hepatocellular adenomas and carcinomas. Previous studies have also indicated that rat liver tumors induced by chemical carcinogens display a considerable increase in the expression of c-myc. A modest increase in the expression of c-Ha-ras was also seen in some of these tumors (30). Regenerating rat liver also displays an increase in the levels of c-myc and c-Ha-ras transcripts (31). These results suggest that increased expression of c-myc in liver tumors may reflect the increased proliferation of these cells and not be a specific marker of the tumor phenotype. Increased expression of the EGF-receptor gene and amplification of this DNA sequence has been described in certain squamous carcinomas and glioblastomas (32, 33), and increased expression of v-raf homologous transcripts has been reported in carcinogen-induced mouse lung and primary rat hepatic tumors (34-36). In this study, we did not find increased expression of either of these sequences in our rat liver tumors and, in fact, several tumors displayed decreased expression of the EGF receptor gene. It is of interest that rat liver tumors induced by diethylnitrosamine were reported to have a decrease in EGF-receptor binding (37).

The present studies indicate that DEN-induced rat liver tumors demonstrate a striking increase in the expression of 2 endogenous retrovirus-related sequences, RaLV and 30S, as well as increased expression of the c-myc oncogene. The extent of expression of the RaLV and 30S sequences in individual tumors did not correlate with the extent of expression of c-myc (compare Figs. 1 and 2). It is also unlikely that the increased expression of the RaLV and 30S sequences is secondary to mutagenic activation of a ras protooncogene since we did not observe increased expression of these sequences in rat fibroblasts transformed by an activated human bladder cancer Ha-ras oncogene, and thus far activated ras oncogenes have not been detected in carcinogen-induced rat liver tumors. Although increased expression of certain endogenous retrovirus-related sequences appears to be a common finding in rat hepatocarcinogenesis, the biological significance of this phenomenon is not clear at the present time. Elsewhere, we have postulated that because of the similarity of certain endogenous retrovirus-related mammalian DNA sequences to transposons in lower organisms, their increased expression in tumors might favor transposition events and thus contribute to the process of tumor progression (3, 6). Studies are in progress to elucidate the underlying mechanism and significance of the present findings.

1 L. L. Hsich and I. B. Weinstein, unpublished studies.

3 M. Barbacid, personal communication.
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