Factors Influencing the Expression of Endogenous Retrovirus-related Sequences in the Liver of B6C3 Mice

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ABSTRACT

The expression of RNA transcripts from three families of endogenous retrovirus-related sequences was investigated during liver cell proliferation in B6C3 mice. Treatment with a single dose of the liver mitogen and promoter of mouse hepatocarcinogenesis 1, 4-bis(3,5-dichloropyridyloxy)benzene (TCPOBOP), or with tetrachloride (CCL4), induced liver cell proliferation at days 2 and 3 after treatment. Both of these treatments led to a marked increase in Moloney murine leukemia virus-related 6 kilobase RNAs, which were most abundant at day 1 after TCPOBOP treatment and at day 2 after CCL4. Intracisternal A particle-related 6-kilobase RNAs were markedly increased at days 1 and 2 after TCPOBOP and at days 1, 2, and 3 after CCL4. VL30-related transcripts were slightly decreased after TCPOBOP, but they were markedly increased at days 1 and 2 following CCL4. The livers of 15-day-old untreated mice contained about a 3-fold higher level of Moloney murine leukemia virus-related RNAs than adult liver. Intracisternal A particle-related 6-kilobase transcripts were present at 3-fold higher abundance in 7-day-old than in 15-day-old or adult liver. RNAs homologous to VL30 were detected at almost the same levels in infant as well as adult livers. Inhibition of protein synthesis by the administration of cycloheximide to adult mice caused a marked increase in the amount of Moloney murine leukemia virus-, intracisternal A particle-, and VL30-related RNAs in the livers of the treated mice, suggesting the existence of labile proteins that normally regulate the abundance of these transcripts. We postulate that the amounts of these putative regulatory proteins vary during both normal development and carcinogenesis and also in response to specific agents that induce liver cell proliferation.

INTRODUCTION

We have recently reported an increase in the expression of Mo-MuLV2- and IAP-related sequences in liver tumors induced by a single dose of diethylaminoamine in male B6C3 mice and also in spontaneously occurring liver tumors in male C3Hf mice (1). The mechanism(s) responsible for constitutive expression of these endogenous retroviruses in liver tumors is not known. The transcription of these sequences might be specific to the tumor phenotype, or it might be related to the high rate of cell proliferation of hepatocellular tumors compared to normal adult liver.

We have, therefore, further investigated the expression of endogenous retroviral families related to Mo-MuLV, IAP, and VL30 sequence during liver cell proliferation induced by TCPOBOP, a liver mitogen and a promoter of mouse hepatocarcinogenesis (2), or by CCL4-induced necrosis and subsequent liver regeneration, or liver proliferation during mouse development.

RESULTS

We have also tested the hypothesis that there exists labile protein repressors that control the expression of these retrovirus sequences, by treating mice with an inhibitor of protein synthesis, cycloheximide. Our results indicate that expression of all three of the retrovirus sequences investigated occurred during liver cell proliferation, although the extent of expression was not simply a function of the extent of cell proliferation. We also found that cycloheximide treatment strongly stimulated transcription of Mo-MuLV and VL30 sequences, suggesting the existence of short-lived protein repressors that normally control the transcription of these sequences.

MATERIALS AND METHODS

Animals and Treatment Regimens. Male and female (C57BL/6J-Dp × C3H/HFrF), (hereafter called B6C3) mice, bred in the laboratories of the Istituto Nazionale Tumori, Milan, Italy, were used. They were fed a standard pellet diet (Piccioni, Brescia, Italy) and water ad libitum. Female mice (9-11 weeks old) were given: a single dose of TCPOBOP (3 mg/kg p.o. by gavage) in a 1% dimethyl sulfoxide/corn oil solution, or simply the dimethyl sulfoxide/corn oil vehicle, or CCL4 (1 ml/kg p.o. by gavage) in corn oil. The mice were sacrificed 1, 2, 3, and 7 days after treatment, and the livers were rapidly excised and frozen. Livers from untreated male mice of 1, 2, and 11 weeks of age were also obtained. Additional groups of three mice each received the above specified treatment schedules and these mice, as well as groups of control mice, were then administered colchicine (25 mg/kg body weight, i.p.), 3 h before sacrifice. Their livers were removed, fixed in Bouin solution, embedded in paraffin, cut, and stained with hematoxylin and eosin. The number of mitoses per 1000 nuclei of hepatocytes was scored on these sections. In another experiment, female mice (15 week old) were treated once with cycloheximide (50 mg/kg i.p.) in 0.9% NaCl solution, and sacrificed 3 h later, or twice with the same dose of cycloheximide, at 0 and 3 h, and sacrificed 6 h later.

Northern Blot Hybridization. Total liver RNA was prepared from the frozen livers using previously described methods (1, 3). The poly(A)+RNA fraction was isolated by passage of the RNA twice through oligo(dT) celluose columns (Collaborative Research, Waltham, MA, or Pharmacia, Uppsala, Sweden) (4).

The following DNA inserts and recombinant plasmids were used: Mo-MuLV: an 8.2-kilobase HindIII fragment excised from plasmid p8.2 (5): IAP: a 5.1-kilobase EcoRI-HindIII fragment excised from plasmid pMIA1, containing internal IAP sequences (6); VL30: a 4.7-kilobase XhoI fragment, including the entire VL30 sequence, contained in the plasmid pVL47 (7); LLRep 3: a 0.8-kilobase PstI fragment contained in the plasmid pLLRep3 (8). Purified fragments and plasmids were 32P-labeled by nick translation (9). Northern blot hybridizations were performed as described previously (1). Methylene blue staining indicated that all lanes of the filters contained approximately equivalent amounts of RNA. The relative abundance of specific transcripts in the different lanes was assayed by densitometric analysis of the autoradiograms.

Effects of TCPOBOP and CCL4 on Hepatocyte Proliferation

The kinetics of liver cell proliferation after a single treatment with TCPOBOP or CCL4, are shown in Fig. 1. Mitoses were not
observed in the control adult livers, nor was there a significant increase 1 day after either of the treatments. Mitotic figures were seen at the highest frequency 2 days after treatment with either TCPOBOP or CCl_4, and this frequency declined at day 3 after treatment. TCPOBOP administration resulted in a smaller number of mitoses/1000 nuclei than CCl_4 treatment (day 2: 25 versus 69 mitoses/1000 nuclei; day 3: 14 versus 17 mitoses/1000 nuclei in TCPOBOP and CCl_4 treated groups, respectively). Seven days after either of the treatments the liver showed almost no sign of mitotic activity. Necrosis was observed in CCl_4 but not TCPOBOP treated livers at days 1, 2, and 3 after treatment.

**Effects of TCPOBOP and CCl_4 on Retrovirus Expression**

Mo-MuLV. RNA transcripts of about 6 kilobase in length, homologous to Mo-MuLV, were present in small amounts in the normal liver and increased 3- and 2-fold, 1 and 2 days after treatment with TCPOBOP, respectively, and then returned to the control level 3 and 7 days after treatment (Fig. 2). RNAs of about 8.4 kilobase, not detected in the control liver, were observed at low abundance 1 day after TCPOBOP treatment. After CCl_4 administration, the abundance of 6-kilobase transcripts increased 9- and 16-fold with respect to the control liver, on days 1 and 2, respectively, and returned to the control level 3 and 7 days after CCl_4 administration. RNA species of 8.4 and 9.4 kilobase were also observed during the first 3 days following CCl_4, and these species were most abundant at day 2 (Fig. 2).

IAP. IAP-related RNAs of about 6 kilobase in length were detected in the control liver, and their abundance was slightly increased by TCPOBOP (2-fold increase at days 1-3 after treatment). CCl_4 caused a much greater increase in the abundance of these RNAs, producing 8-, 4-, and 4-fold increases at days 1, 2, and 3 after treatment, respectively.

VL30. The quantity of a 5.5-kilobase transcript homologous to the VL30 probe was slightly decreased after treatment with TCPOBOP, but it was increased up to 14-fold 1 day after CCl_4 treatment and about 4-fold at day 2 following CCl_4 treatment and then returned to the control level 3 and 7 days after CCl_4 treatment. Transcripts of 8.2 and 9.4 kilobase in size were also observed at day 1 after CCl_4 treatment (Fig. 2).

**Retrovirus Expression in Infant Liver**

Mo-MuLV-related transcripts, 6 kilobase in size, were seen at lower abundance in the livers of 7-day-old mice than in the adult liver. However, the livers of 15-day-old animals contained about a 3-fold excess of this RNA species with respect to the adult liver. In addition, RNA from the livers of 15-day-old mice displayed Mo-MuLV-related species of 8 and 8.8 kilobase, which were only barely detected in the liver samples from 7-day-old and adult mice. For additional details see "Materials and Methods."

Parallel studies on hepatocyte proliferation indicated that in untreated B6C3 male mice the number of mitoses per 1000 nuclei (mean ± SE) on day 7, day 15, and at 11 weeks of age were 12.3 ± 1.2, 9.3 ± 0.9, and 0, respectively. Thus, although the IAP-related transcript was present at highest levels on day 7, when the mitotic index was also highest, there was no simple correlation between the abundance of specific Mo-MuLV- and VL30-related transcripts and mitotic index, during normal development.

**Cycloheximide Treatment of Adult Mice**

When the RNAs were obtained from the livers of adult mice treated with cycloheximide, either 3 h after a single dose or 6 h after two doses repeated at 3 h intervals, they revealed a marked increase in the abundance of 6-kilobase Mo-MuLV-related RNAs (11-fold increase with respect to control livers), a marked increase in the abundance of a 5.5-kilobase transcript homologous to VL30 (16- and 14-fold stimulation at 3 and 6
Fig. 3. Northern blot analysis of Mo-MuLV-, IAP, VL30-, and LLRep3-related RNAs in liver RNA samples obtained from infant and adult mice. For additional details see “Materials and Methods.”

Days 7, 15, adult

Endogenous Retrovirus Sequences in Mouse Liver

h, respectively), and a slight increase in the abundance of 6-kilobase IAP-related sequences (2- and 3-fold stimulation at 3 and 6 h, respectively) (Fig. 4). Histological examination of liver samples on days 1 and 2 after administration of these doses of cycloheximide revealed no evidence of hyperplasia, necrosis, or other gross evidence of toxicity.

Hybridization with a Probe for a “House Keeping” Gene

Hybridization of filters with a probe for a mouse repetitive sequence (LLRep3) isolated from a liver cDNA library (8) showed that the abundance of LLRep3-related transcripts was not affected by TCPOBOP, CCl4, cycloheximide, nor by the age of the animals (Figs. 2-4).

Discussion

The kinetics of mitotic activity of hepatocytes after in vivo treatment with either TCPOBOP or CCl4 followed a similar pattern, although CCl4 caused about a 3-fold greater increase in mitotic activity on day 2 after treatment than that observed with TCPOBOP. The mechanism of stimulation of hepatocyte proliferation by TCPOBOP and CCl4 are apparently different, since CCl4 induces necrosis and subsequent regeneration, while TCPOBOP stimulates hepatocyte proliferation without evidence of necrosis or cytotoxicity.

We have observed that the expression of specific endogenous retrovirus-related DNA sequences increases during liver cell proliferation in adult mice. We found that 6 kilobase Mo-MuLV-related transcripts were most abundant at day 1 after TCPOBOP treatment and at day 2 following CCl4 treatment. The abundance of 6 kilobase IAP-related RNAs was increased at days 1 and 2 after TCPOBOP, and at days 1, 2, and 3 after CCl4 treatment. VL30-related transcripts were slightly decreased after TCPOBOP, but they were strongly increased at days 1 and 2 following CCl4 (Fig. 2). Although cell proliferation may play a role, the increased abundance of these RNAs does not appear to be simply a consequence of cell proliferation since an increase in these RNAs was already apparent by day 1 following drug treatment, and the increase in mitotic index was not apparent until day 2. Our studies on the abundance of these RNAs in infant liver (Fig. 3) and comparisons with mitotic activities during development also suggest that other factors control the expression of these RNAs. We did not investigate possible alterations in abundance of retroviral-related transcripts in specific cell types and, therefore, liver infiltration by mononuclear cells present only in CCl4-treated mice may play a role in the difference observed between the TCPOBOP and CCl4-treated groups (Fig. 2).

A striking finding in the present studies is that the treatment of adult mice with cycloheximide stimulated the expression of Mo-MuLV-, IAP-, and VL30-related sequences, in the livers of these animals (Fig. 3). These findings suggest that labile repressor proteins may normally act to inhibit the expression of these sequences. The enhancement observed with cycloheximide was greater on the Mo-MuLV- and VL30-related RNAs than on the IAP-related RNAs. A previous study indicated that cycloheximide induces type C retrovirus replication and transcription in mouse embryo fibroblasts in culture (10). The stronger effect of CCl4 than TCPOBOP in increasing the expression of Mo-MuLV and VL30 RNAs is consistent with the effects of cycloheximide because CCl4 treatment also inhibits protein synthesis in the liver (11), whereas TCPOBOP actually stimulates protein synthesis (12). The peak in Mo-MuLV-related RNA species observed in 15-day-old liver (Fig. 3) may be due to a decrease of the level of the putative repressor proteins at that stage of development. Obviously, further studies are required to determine whether the increased abundance of these retrovirus-related RNAs in the livers of adult mice treated with TCPOBOP or CCl4, and during normal liver development, reflect increased de novo transcription or decreased turnover of
the related RNAs. Within this context, the putative labile repressor proteins might act either as inhibitors of transcription or enhancers of RNA degradation. The existence of labile repressor proteins is further indicated by the experiment showing no alteration in abundance of the "housekeeping" LLRep3-related transcripts by cycloheximide treatment. Therefore, stimulation of retrovirus transcription cannot be attributed to a nonspecific effect of cycloheximide on RNA catabolism.

The endogenous retroviral families investigated in the present study are present in the mouse genome in multiple copies (about 30–50 copies of both Mo-MuLV- and VL30-related sequences, and about 1000 of IAP-related sequences per cell). Within these retroviral families, members or subfamilies are present that vary in genome size (13, 14). We have observed a low level of transcription of Mo-MuLV- and IAP-related RNAs of 6 kilobase in size in the normal adult liver and RNA species of the same length were induced by CCl₄ and cycloheximide treatment. The treated animals also displayed, however, some expression of higher molecular weight transcripts (Fig. 2) presumably indicating a switch-on in the expression of otherwise inactive subfamilies, or alterations in the transcription of the 6 kilobase genomes.

We have previously reported that carcinogen-induced mouse liver tumors display constitutive high-level expression of Mo-MuLV, and IAP-related RNAs, of the same size as those seen in the present study (1). The constitutive expression of these RNAs in these tumors might be due to an absence or deficiency of the putative repressor proteins discussed above, due to suppression of their expression or mutations in the corresponding genes. The putative repressor proteins regulating the transcription of Mo-MuLV, IAP-, and VL30-related DNA sequences appear to be different from each other, because the patterns of expression of the individual retroviral sequences differ in various situations, i.e., during normal development, after treatment with TCPOBOP or CCl₄, and between individual hepatocellular tumors (Ref. 1, and the present studies).

Finally, if the above-mentioned putative repressor proteins also regulate the levels of expression of other cellular genes, their lack of function in liver tumors may account for the widespread alterations in gene expression that are commonly observed in liver tumors (15, 16). Thus, studies on the mechanisms that regulate the expression of endogenous retrovirus-like DNA sequences in normal liver and in liver tumors may provide insights into the more widespread disturbances in gene expression associated with neoplasia.

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