Volume 15 Number 21 1987 Nucleic Acids Research

Cloning and characterization of a human ribosomal protein gene with enhanced expression in fetal and neoplastic cells

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Received May 5, 1987; Revised and Accepted October 5, 1987

ABSTRACT

Hepatocellular carcinoma is strongly associated with hepatitis B virus carrier patients who usually have HBV sequences integrated in the chromosomal DNA of liver cells. To assess the possible effects of HBV regulatory sequences (e.g., the enhancer) on expression of neighboring host genes we have screened for cellular genes that are both overexpressed and adjacent to integrated HBV sequences in hepatocellular carcinoma cells. The cloned cDNA for one such gene encodes a protein similar to the E. coli L-3 ribosomal protein which is thought to play a role in mRNA binding to the ribosome. The protein encoded by the cDNA localizes to the nucleolus and is also found in ribosomes; possibly it is the mammalian homologue of L-3 (MRL3). The expression of MRL3 is higher in colon carcinoma and lymphoma cell lines than in normal liver, placenta and diploid fibroblasts, and is also higher in fetal than in adult liver. Therefore, MRL3 overexpression seems to be a property of rapidly dividing cells and is not directly linked to oncogenesis.

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic hepatitis in humans and is causally linked to hepatocellular carcinoma (HCC), the world's most prevalent cancer (1). HBV DNA is frequently integrated into the host DNA in the liver of carrier patients (2) who have a dramatically increased predilection to HCC (1). Furthermore, integrated viral sequences are present in a high proportion of HCC's (3). Since HBV does not appear to carry any acutely transforming oncogene (4), it is possible that the integration of HBV DNA somehow plays a role in oncogenesis.

We and others have characterized the integrated HBV DNA in the HCC cell line PLC/PRF/5 (Alexander cells) (5) through molecular cloning and sequence analysis (6-10). There are at least seven integrated fragments of HBV DNA in the Alexander cell genome; there are no full-length copies and most of the fragments are highly rearranged. In at least two instances there has been secondary translocation of integrated sequences to new sites in the chromosome (10,11). Further, there is evidence of mutagenesis of both HBV and proximal host sequences (8,11,12; Garcia et al., unpublished). It is not known whether

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these rearrangements and mutations play a role in tumorigenesis. However, they are not required for oncogenesis since some HCC's have a single full-length integrated HBV sequence (13).

We have tested for the possible activation by promoter-readthrough of cellular oncogenes by screening for hybrid RNA species containing HBV sequences linked to host coding regions. None of the isolated hybrid RNA species contained sufficient linked coding sequences to represent a strong candidate for an oncogenic sequence (12). An alternative possibility could involve the HBV enhancer element (14,15) that could in principle activate host genes in the vicinity of integrated HBV DNA. We have therefore screened for genes that are overexpressed and are located near one of the integrated HBV DNA fragments carrying the HBV enhancer. We report here the identification of a gene that is overexpressed in HCC cells. This gene encodes a ribosomal protein which shows sequence similarity to the bacterial ribosomal protein L3, and hereafter will be referred to as the MRL3 (mammalian ribosome L3) gene. The expression of this gene is augmented not only in HCC cells but also in other neoplastic cells as well as fetal liver cells.

MATERIALS AND METHODS

Southern Blotting for the Identification of Activated Genes

DNA prepared from lambda phage clones containing integrated HBV DNA previously isolated from a genomic library of Alexander cells was digested with EcoRI and Southern blotted. Labelled cDNA probes were prepared from the polyA+ RNA of either normal liver or HCC cells as follows: 2.5 μg polyA+ RNA were incubated with 15 μg of calf thymus random primers for 5 min at 65°C in H_2O. The reverse transcription reaction was carried out in 50 μl of RT buffer (50mM Tris-HCl, pH 8.2, 50mM KCl, 2mM dithiothreitol, 8mM $MgCl_2$) with 4mM sodium pyrophosphate, 1mM each of dATP, dGTP and dTTP, 250 μCi α-^{32}P-dCTP and 32 units of reverse transcriptase (Life Sciences) at 42°C for 20 min. The reaction was chased with 0.1mM dCTP for 1 hr. The probe prepared in this way contained about $1x10^7$ Cerenkov cpm/μg. Hybridization was performed at 42°C in 5xSSC containing 3x Denhardt's solution, 50% formamide and 100 μg/ml salmon sperm DNA. The blot was washed in 0.1xSSC, 0.1% SDS at 50°C.

Preparation and Screening of the cDNA Library

An Alexander cell cDNA library was constructed in the λgt10 vector (16) using an oligodT primer, while a normal human liver cDNA library was constructed using a λgt11 vector (16) using calf thymus DNA random primers. For the synthesis of the first strand cDNA, 10 μg of the polyA+ RNA doubly purified on

an oligodT column (Collaborative Research) was incubated with 8 µg oligo-dT_{12-18} (Pharmacia) (or 56 µg random primer), 100 units RNAsin (Promega Biotec), 1mM deoxyribonucleotides (dNTP's), 100 µCi α-^{32}P-dCTP, 100 units of reverse transcriptase in 200 µl of RT buffer at 42°C for 1 hr. The reaction was stopped by adding EDTA to a final concentration of 20mM and the mixture was extracted with phenol/$CHCl_3$. The aqueous phase was precipitated with ethanol in the presence of 2M ammonium acetate and the precipitate was resuspended in 100 µl of 0.1N NaOH and incubated at 65°C for 1 hr. The cDNA was neutralized with 2.5 µl of 4N HCl and 5 µl of 2M sodium acetate, pH 5.6 and purified by ethanol precipitation. In preparing the oligodT primed library, the second strand was synthesized using 45 units of the Klenow fragment of DNA polymerase I (Boehringer Mannheim) in 200 µl of NT buffer (50mM Tris-HCl, pH 7.2, 10mM $MgCl_2$, 1mM DTT, 500 µg/ml bovine serum albumin), containing 1mM each of dNTP's at 14°C overnight. The reaction was stopped by EDTA. Phenol/$CHCl_3$ extraction and ammonium acetate precipitation was carried out as in the first strand reaction. To complete the second strand synthesis, the cDNA was further treated at 42°C for 1 hr with 100 units of reverse transcriptase in 200 µl RT buffer containing 1mM of dNTP's. The reaction was stopped and the cDNA isolated as described above. In preparing the randomly primed normal liver library, the first strand was tailed with oligodT by incubation for 5 min at 37°C in a reaction mixture of 100 µl containing 0.1mM TTP, 1mM $CaCl_2$, 1mM β-mercaptoethanol, 140mM sodium cacodylate, 30mM Tris-HCl, pH 7.6, and 60 units of terminal deoxynucleotidyl transferase (New England Nuclear). The second strand synthesis was carried out using an oligodA primer (4 µg, Pharmacia) in 100 µl RT buffer containing 1mM dNTP's and 50 units of reverse transcriptase at 42°C for 1.5 hr. The synthesis was repeated with 10 units of Klenow fragment in 100 µl NT buffer containing 1mM dNTP's at room temperature for 1 hr. The double stranded cDNA was subsequently digested with 100 units of nuclease S1 (Pharmacia) in 100 µl of solution containing 30mM sodium acetate, pH 4.6, 150mM NaCl, 1mM $ZnSO_4$ at room temperature for 30 min. The reaction was stopped with EDTA, and the cDNA prepared as described above. The cDNA was methylated in 100 µl reaction mixture containing 50mM Tris-HCl, pH 7.5, 1mM EDTA, 5mM DTT, 5 µM S-adenosyl-methionine and 20 units of EcoRI methylase (New England Biolabs) at 37°C for 30 min. After phenol extraction and ethanol precipitation the methylated DNA was further treated with DNA polymerase I at room temperature for 1 hr. Linker ligation, EcoRI digestion, and size fractionation on Biogel A-150m were done as previously described (17). The random primed library was size fractionated on a 1% agarose gel.

DNA fragments larger than 600 bp were pooled. Ligation of cDNA fragments to the vector and the subsequent screening of the library were carried out as described by Ebina et al. (17). The hybridization stringency for screening cDNA libraries was the same as described above for Southern blots. Two Alexander cDNA clones, pGT1 and pGT2, containing the entire coding sequence of the MRL3 gene were isolated. Three different overlapping clones which together comprise the entire reading frame of the MRL3 gene were also isolated from the random-primed, normal human liver cDNA library. A cDNA clone, pRR, which contains the entire coding sequence of the rat MRL3 gene, was isolated from a BRL cell cDNA library (kindly provided to us by Dr. J. Edman) using the same hybridization stringency and the nick translated human cDNA clone pGT1 as the probe.

RNA Dot Hybridization

RNA in 10 µl 50% formamide, 2.2M formaldehyde, 10mM Na phosphate, pH 7, 1mM EDTA, 5mM Na acetate was heated briefly at 65°C. After the addition of 90 µl 15xSSC, the RNA was spotted on the nitrocellulose filter with a manifold (Schleicher & Schuell). Hybridization was carried out with nick translated cDNA probes as described above.

Expression in E. coli of MRL3 as a Beta-Galactosidase Fusion Protein

The SalI/EcoRV double-digested fragment of pGT1 (Fig. 3, bracketed) was inserted into the expression vector pUR292 (18) at the SalI/HindIII site, and then transfected into E. coli DG101. Positive transformants were selected by the presence of a new protein of the expected molecular weight after induction with isopropyl-β-D-thiogalactopyranoside. The MRL3-β-galactosidase fusion protein was purified by a modification of the method of Yen & Webster (19). Briefly, the cells were grown in Luria broth, treated with 1mM isopropyl-β-D-thiogalactopyranoside for 2 hr and then harvested by centrifugation, resuspended in 10mM Tris-HCl, 1mM EDTA, pH 8.0, with 0.5 mg/ml lysozyme (Sigma), and sonicated on ice for 5 min. The mixture was centrifuged at 7000 rpm in a Sorvall GS3 rotor for 10 min and the pellet was dissolved in Laemmli electrophoresis buffer and separated by electrophoresis through a 7% polyacrylamide gel. The proteins were stained with 0.1% Coomassie Blue R-250 in water, and the fusion protein band was cut out and electroeluted overnight in an Isco Electroelutor. The purity of the protein was checked by analytical polyacrylamide gel electrophoresis.

Immunochemical Studies

Approximately 200 µg of the purified β-galactosidase-MRL3 fusion protein in complete Freund's adjuvant were injected into each of two New Zealand white

rabbits. After four weeks, the rabbits were boosted at two-week intervals with 100 µg of the fusion protein in incomplete Freund's adjuvant. Sera from the rabbits were tested for their specificity against MRL3 protein by immunoprecipitation experiments using ^{35}S-methionine-labeled MRL3 protein synthesized in vitro from MRL3 mRNA transcribed off the SP6 phage derived vector, pSP65 (Promega Biotech) (20).

For preparation of monoclonal antibodies, spleen cells from Balb/c mice previously immunized with the β-galactosidase-MRL3 fusion protein were fused with SP2/0 myeloma cells (21). Hybridomas were isolated by standard techniques (21), and screened by paired enzyme immunoassay (22) against β-galactosidase and the fusion protein. Three clones which produced antibody reacting with the MRL3 fusion protein but not with β-galactosidase were further characterized. One of these, pXG6, an antibody of the IgG2a subclass, was highly efficient in immunoprecipitation of MRL3 protein synthesized _in vitro_ (Fig. 7A), and was used for further analysis.

Western blotting and immunoprecipitation were performed as described previously (19). For immunohistochemistry, Mahlavu hepatoma cells, grown on coverslips to near-confluence were washed twice with PBS, fixed in ice-cold acetone for 10 min and dried. After preincubation in PBS with 1% (w/v) bovine serum albumin for 1 hr, the coverslips were treated with hyperimmune rabbit serum, diluted 1:25 in PBS + 1% albumin. After 2 hr, the coverslips were washed with PBS 3 times and incubated for 1 hr with fluorescein-conjugated goat anti-rabbit IgG (Sigma), diluted 1:50 in PBS + 1% albumin. The coverslips were washed again with PBS, mounted on glass slides with 50% glycerol, and examined with a fluorescent microscope.

RESULTS

Strategy for Searching Activated Genes Flanking Integrated HBV DNA in Alexander Cells

13 distinct lambda phage clones containing integrated HBV DNA have been isolated by our laboratory from the Alexander cell genomic library; some of these phage clones contain overlapping host sequences (10,11). These clones may contain the entire complement of HBV containing DNA fragments. The strategy for searching for activated host genes flanking the HBV DNA is described in Experimental Procedures. Briefly, the EcoRI fragments derived from these phage clones were analyzed by Southern blotting with single-stranded, radioactively labeled cDNA probes prepared from polyA+ RNA of either normal human liver or hepatoma tissues. Many restriction fragments

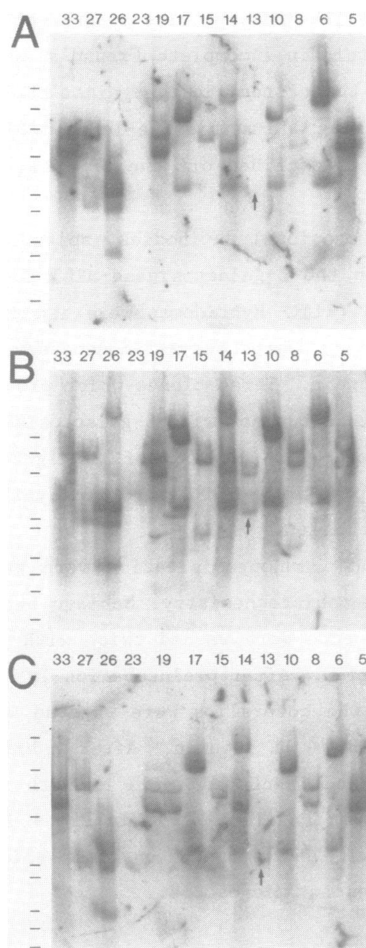

FIGURE 1. Identification of activated genes located near integrated HBV DNA. Details of the experiments are described in Experimental Procedures. The numbers indicate the various genomic phage clones previously isolated in our lab (9,10). Phage DNA digested with EcoRI and blotted to nitrocellulose was hybridized with cDNA probes prepared from A, normal human liver; B, Alexander HCC cell line (5); C, Mahlavu HCC cell line (30). Arrows denote the restriction fragment that hybridizes to Alexander and Mahlavu cDNA probes but not to normal liver cDNA probes. Some restriction fragments hybridize only to Alexander cDNA probes and not to normal liver or Mahlavu probe. These fragments contain HBV sequences and are presumably hybridized to the cDNA probes derived from the HBV mRNA in Alexander cells (12). The sizes of the molecular weight markers as denoted next to the gels are from top to bottom, 23K, 9.5K, 6.5K, 4.3K, 2.3K, 2.0K, 1.3K, 1.0K, 872 and 603 bases.

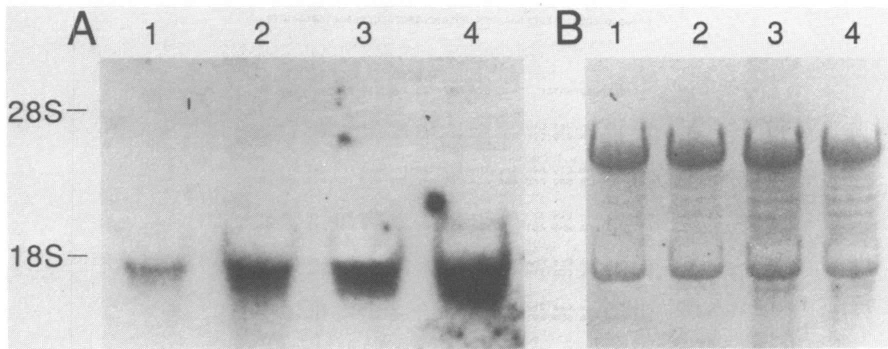

FIGURE 2. Northern analysis of the MRL3 mRNA. (A) 10 µg of total cellular RNA were loaded on each lane and blotted to nitrocellulose after electrophoresis. Both pGT1 and pGT2 probes give the same hybridization pattern. Lane 1, normal human liver; lane 2, HCC tissue from surgical excision; lane 3, Alexander cell line; lane 4, Mahlavu cell line. Tissues in lane 1 and lane 2 were isolated from the same patient. (B) The same gel stained with ethidium bromide.

hybridized to the cDNA (Fig. 1). A detailed analysis of the hybridization with one of the restriction fragments indicated that the hybridization was due, in part, to the presence of repetitive sequences present in polyA+ RNA (data not shown). Nevertheless, it was possible to identify an EcoRI fragment of clone 13 that hybridized specifically to the cDNA probes prepared from two different HCC cell lines but not to the probe prepared from normal liver (Fig. 1, denoted by an arrow). Thus this sequence is apparently actively transcribed in hepatoma cells but less so in normal liver cells.

The cDNA of MRL-3

This EcoRI fragment was further digested with EcoRV to generate two fragments 1.6 kb and 1 kb in length. The 1.6 kb fragment contained low frequency repetitive DNA sequences and therefore could not be employed as a probe. The 1 kb fragment did not contain repetitive sequences and was used to screen a cDNA library prepared from Alexander cell polyA+ RNA. Two related cDNA clones (pGT1 and pGT2), 1.6 kb and 1.5 kb in length, respectively, were isolated. Both of these cDNA clones hybridize to a major RNA species of 1.8 kb. As shown in Figure 2, this gene is expressed in both normal liver and hepatoma cells, but the level of expression (normalized to ribosomal RNA) is three to five fold higher in HCC cells as compared to normal liver cells.

The nucleotide sequence (23) of these two cDNA clones is presented in

```
GGTGGCGTGGGGACTCCCTGAAAGCAGAGCGGCAGGGCGCCCGGAAGTCGTGAGTCGAG

                          1                                     10
                         Met Pro Gly Trp Arg Leu Leu Thr Gln Val
TCTTCCCGGGCTAATCC        ATG CCG GGT TGG AGG CTG CTG ACG CAG GTC

                                             20
Gly Ala Gln Val Leu Gly Arg Leu Gly Asp Gly Leu Gly Ala Ala
GGC GCC CAG GTG CTG GGT CGA CTC GGG GAC GGC CTG GGT GCT GCC

                30                                          40
Leu Gly Pro Gly Asn Arg Thr His Ile Trp Leu Phe Val Arg Gly
CTG GGC CCG GGG AAC AGA ACA CAC ATC TGG CTT TTT GTT AGA GGT

                                             50
Leu His Gly Lys Ser Gly Thr Trp Trp Asp Glu His Leu Ser Glu
CTT CAT GGA AAG AGT GGT ACA TGG TGG GAT GAG CAT CTT TCT GAA

                60                                          70
Glu Asn Val Pro Phe Ile Lys Gln Leu Val Ser Asp Glu Asp Lys
GAA AAT GTC CCA TTC ATT AAG CAG TTG GTC TCT GAT GAA GAT AAA

                                             80
Ala Gln Leu Ala Ser Lys Leu Cys Pro Leu Lys Asp Glu Pro Trp
GCC CAA TTA GCA AGT AAA CTG TGT CCT CTG AAA GAT GAA CCA TGG

                90                                         100
Pro Ile His Pro Trp Glu Pro Gly Ser Phe Arg Val Gly Leu Ile
CCT ATA CAT CCT TGG GAA CCA GGT TCC TTT AGA GTT GGT CTT ATT

                                            110
Ala Leu Lys Leu Gly Met Met Pro Leu Trp Thr Lys Asp Gly Gln
GCC TTG AAG CTG GGC ATG ATG CCT TTA TGG ACC AAG GAT GGT CAA

               120                                         130
Lys His Val Val Thr Leu Leu Gln Val Gln Asp Cys His Val Leu
AAG CAT GTG GTC ACA TTA CTT CAG GTA CAA GAC TGT CAT GTC TTA

                                            140
Lys Tyr Thr Ser Lys Glu Asn Cys Asn Gly Lys Met Ala Thr Leu
AAA TAT ACG TCA AAG GAA AAC TGT AAT GGA AAA ATG GCA ACC CTG

               150                                         160
Ser Val Gly Gly Lys Thr Val Ser Arg Phe Arg Lys Ala Thr Ser
TCT GTA GGA GGA AAA ACT GTA TCA CGT TTT CGT AAA GCT ACA TCC

                                            170
Ile Leu Glu Phe Tyr Arg Glu Leu Gly Leu Pro Pro Lys Gln Thr
ATA TTG GAA TTT TAC CGG GAA CTT GGA TTG CCG CCG AAA CAG ACA

               180                                         190
Val Lys Ile Phe Asn Ile Thr Asp Asn Ala Ala Ile Lys Pro Gly
GTT AAA ATC TTT AAT ATA ACA GAT AAT GCT GCA ATT AAA CCA GGC

                                            200
Thr Pro Leu Tyr Ala Ala His Phe Arg Pro Gly Gln Tyr Val Asp
ACT CCT CTT TAT GCT GCT CAC TTT CGT CCA GGA CAG TAT GTG GAT

               210                                         220
Val Thr Ala Lys Thr Ile Gly Lys Gly Phe Gln Gly Val Met Lys
GTC ACA GCC AAA ACT ATT GGT AAA GGT TTT CAA GGT GTC ATG AAA

                                            230
Arg Trp Gly Phe Lys Gly Gln Pro Ala Thr His Gly Gln Thr Lys
AGA TGG GGA TTT AAA GGC CAG CCT GCT ACG CAT GGT CAA ACG AAA

               240                                         250
Thr His Arg Arg Pro Gly Ala Val Ala Thr Gly Asp Ile Gly Arg
ACC CAC AGG AGA CCT GGA GCT GTT GCA ACT GGT GAT ATT GGC AGA

                                            260
Val Trp Pro Gly Thr Lys Met Pro Gly Lys Met Gly Asn Ile Tyr
GTC TGG CCT GGA ACT AAA ATG CCT GGA AAA ATG GGA AAC ATA TAC

               270                                         280
Arg Thr Glu Tyr Gly Leu Lys Val Trp Arg Ile Asn Thr Lys His
AGG ACA GAA TAT GGA CTG AAA GTG TGG AGA ATA AAC ACA AAG CAC

                                            290
Asn Ile Ile Tyr Val Asn Gly Ser Val Pro Gly His Lys Asn Cys
AAC ATA ATC TAT GTA AAT GGC TCT GTA CCT GGA CAT AAA AAT TGC

               300                                         310
Leu Val Lys Val Lys Asp Ser Lys Leu Pro Ala Tyr Lys Asp Leu
TTA GTA AAG GTC AAA GAT TCT AAA CTG CCT GCA TAT AAG GAT CTC

                                            320
Gly Lys Asn Leu Pro Phe Pro Thr Tyr Phe Pro Asp Gly Asp Glu
GGT AAA AAT CTA CCA TTC CCT ACA TAT TTT CCT GAT GGA GAT GAA

               330                                         340
Glu Glu Leu Pro Glu Asp Leu Tyr Asp Glu Asn Val Cys Gln Pro
GAG GAA CTG CCA GAA GAT TTG TAT GAT GAA AAC GTG TGT CAG CCC

                                   348
Gly Ala Pro Ser Ile Thr Phe Ala OC
GGT GCG CCT TCT ATT ACA TTT GCC TAA CATCTTTGGACGTGGCAGAACCTT

ACATATTCTGTGAGCTTCGATGAGCCAGAGTGATATCATAACCACCAGAAATCATACTCT

CCTTTCTTAGTCACAACAAAATCACACATGTCATCTTTGTCAAGGGCATAAATATATCAT

TCATACCCCCATTAAATTTTGTTAGAAAAATTACCACATTAAATATATGAGTTAAGTAGA

          *
TTGGATTTGCTGAAATTGGTGTTGGGCATATTAGCAAAATATTCTTAATTTGTGGACTCG

ATTCTTTTTTACTACATATTTCCCAAGTTATCTTAAGATGTCTGTAAATTTAACTTTTAT

TAAAGTTTTGTCAATCTTTGTGAAATAGTGGTTGTGGAACAGTAGAAAACCATATGGGGA

CTATAGTGCAACCTATTTGGGTAAAGAAACCATTTGCTAAAATGGAGAAAGTAAATAGAT

TTTTATTTAAATTACAGAAACATGTTAAAGGCCGGACAAAGGAAAGACAATAAAATCATA

     *
AATTATC
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Figure 3. The sequence contains an open reading frame of 1047 nucleotides which could encode a protein of 38K daltons. The deduced amino acid sequence shows significant similarity (62 identical residues plus 21 homologous residues) to the E. coli ribosomal protein L3 (24) (Fig. 4). The most similar region (Fig. 4, boxed) extends over a stretch of 40 amino acids and contains more than 50% identical residues (over 70% including homologous substitutions). Therefore, this molecule could be the mammalian homologue of L3 and we termed it MRL3. However, this protein is considerably larger than L3: it contains an additional 96 amino acid residues at the amino terminal end, and an additional 43 residues at the carboxyl terminus, 6 of which are prolines and 10 are aspartate and glutamate residues. It is conceivable that some of the additional sequences serve as a signal for transport into the nucleus, but there is no obvious similarity with known nuclear transport sequences.

Preliminary blot experiments with genomic DNA indicated that this gene is highly conserved among mammals. We have thus isolated several related cDNA clones from a rat liver cell cDNA library using the human cDNA probe. The amino acid sequence deduced from the nucleotide sequence of the rat cDNA clones (Figure 4) shows that there is about 84% sequence identity between the rat and human proteins.

In order to determine whether the coding sequence of the MRL3 gene is mutated in the HCC cells, several cDNA clones were isolated from a normal human liver cDNA library and were sequenced. No sequence variations were found between the normal liver and the HCC cell. Thus, the protein is apparently not mutated during HCC oncogenesis.

The MRL3 Gene is Expressed at High Levels in Tumor Tissues and in Developing Tissues

To test whether the activation of the expression of this gene is HCC-specific, we determined the relative levels of MRL mRNA in various human tumor cells including HCC, lymphoma and adenocarcinoma, versus that in non-transformed cells (full-term placenta, liver and cultured diploid fibroblasts). As shown by dot blot hybridization (Figure 5B), the MRL3 gene is overexpressed 2-6 fold in all the neoplastic cells. When polyA+ RNA was used in a similar Northern blot, the difference appeared even more striking (less than 10 fold).

FIGURE 3. The nucleotide sequence of the MRL3 gene. The polyadenylation sites of pGT1 and pGT2 are denoted by asterisks. The sequence used for expression in E. coli is bracketed. The deduced amino acid sequence is shown on top of the nucleotide sequence.

```
Human   MPGWRLLTQVGAQVLGRLGDGLGAALGPGNRTHIWLFV-RGLHGKSSTWWDEHLSEENVPFIKQLVSDED
Rat     MPGWRLLAQGGAQVLGGGAGGLGAAPGLGSRKNI-LFVVRNLHSKSSTWWDEHLSEENVSFVKQLVSDEN

Human   KAQLASKLCPLKDEFWPIHPWEPGSFRVGLIALKLGMMPLWTKDGQKHVVTLLQVQDCHVLKYTSKEDCN
Rat     KAQLTSLLNPLKDEFWPLHPWEPGSSRVGLIALKLGMMPLWTKDGQKHAVTLLQVQDCHVLKYTPKEDHN
E.coli                                MIGLVGKKVGMTRIFTEDGVSIPVTVIEVEANRVTQVKDLANDG

Human   GKMATLSVGGKTVSRFRKATSILEFYRELGLPPKQTVKIFNITDNAAIKPGTPLYAAHFRPGQYVDVTAK
Rat     GKTATLTVGGKTGSRLYKANSILEFYRDLGLPPKQTTKIFHVTDNAVIKQGTPLYAAHFRPGQYVDVTAK
E.coli  YRAIQVTTGAKKANRVTKPEA--GHFAKAGVEAGRGLWEFRLAEGEEFTVGQSISVELFADVKKVDVTGT

Human   TIGKGFQGVMKRWGFKGQPATHGQTKTHRRPGAVATGDI-GRVWPGTKMPGKMGNIYRTEYGLKVWRINT
Rat     TIGKGFQGVMKRWGFKGQPASHGQTKTHRRPGAISTGDI-ARVWPGTKMPGKMGNQNRTVYGLKVWRVNT
E.coli  SKGKGFAGTVKRWNFRTQDATHGNSLSHRVPGSIGQNQTPGKVFKGKKMAGQMGNERVTVQSLDVVRVDA

Human   KHNIIYVNGSVPGHKNCLVKVKDSKLPAYKDLGKNLPFPTYFPDGDEEELPEDLYDENVCQPGAPSITFA
Rat     KHNIIYVNGSV-GHRNCLVKIKDSTLPAYKDLGKSLPFPTYFPDGDEEELPEDLYDESVRQPSDPSITFA
E.coli  ERNLLLVKGAVPGATGSDLIVKPAVKA
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FIGURE 4. Comparison of the human and rat MRL3 and E. coli ribosomal protein L3 sequences. Lines indicate identical residues, while colons indicate conservative replacements. The highly conserved region is boxed. The E. coli sequence was from Muranova et al. (21). Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Iso; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

The developmental regulation of MRL3 gene expression was studied using the full-length rat cDNA clone, pRR, as a probe. As shown in Figure 5A, this gene is expressed in fetal and newborn livers at a level three to five times higher than in the adult liver. Consistent with the results from the human cells (Figure 2), this gene is also overexpressed (4-5 fold) in rat hepatoma cells that have been generated by a mechanism not involving an hepadna virus.

The MRL3 Gene Encodes a Ribosomal Protein

We confirmed the ribosomal location of this gene product by immunocytochemical methods (see Experimental Procedures). The immunofluorescence staining of Mahlavu hepatoma cells using hyperimmune serum against MRL3 protein is shown in Figure 6. Although the staining in the cytoplasm is obscured by a high background, the staining in the nuclei is clearly restricted to the nucleolus. This result is further confirmed by protein blotting experiments, which show that this protein is present in both the nuclear and ribosomal fractions of HeLa cells, but not in the post-ribosomal supernatant (Fig. 7B). Others have shown that the mRNA level of several ribosomal protein genes decreases 1.5 to

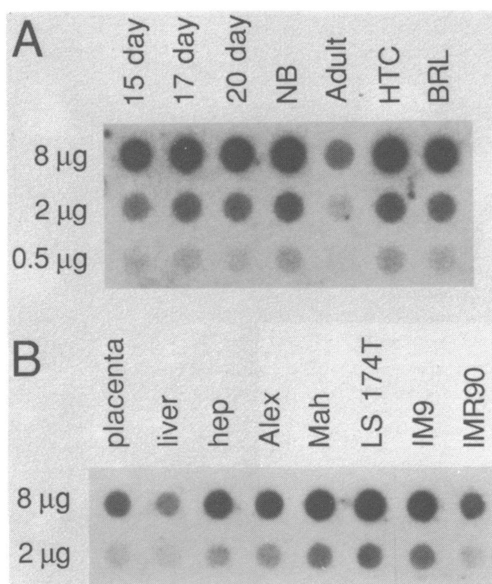

FIGURE 5. (A) Developmental profile of MRL3 mRNA. The rat cDNA clone, pRR, was used as the probe. 8 µg, 2 µg or 0.5 µg of total RNA was spotted on the filter. RNA was isolated from rat fetal liver (15 day, 17 day or 20 day) tissues, or HTC or BRL cells (two different rat HCC cell lines). (B) Dot hybridization of RNA from various human cells using the MRL3 cDNA probe. 8 µg or 2 µg of total RNA from full-term placenta, normal human liver, HCC tissue (hep), Alexander cell line (Alex), Mahlavu cell line (Mah), adenocarcinoma (LS174T) cell line, lymphoma (IM9) cell line, and diploid fibroblast (IMR90) cell line was used for hybridization. The hepatoma tissue was isolated from the same patient that provided the normal liver tissue. Cell lines were obtained from the ATCC, except for Alexander (5) and Mahlavu (30) cells.

2 fold in myoblasts after differentiation (25). Consistent with these findings we find lower levels of MRL3 mRNA in fully differentiated cells.

The MRL3 Promoter is Far Upstream from the Integrated HBV Sequence

Southern blotting of EcoRI digested Alexander cell genomic DNA using the cDNA clones as probes revealed five bands (~8.5 kb, ~8 kb, ~2.5 kb, ~2 kb, ~1.5 kb) with a combined length of ~22 kb (Fig. 8A). Similar Southern blotting of a genomic phage clone revealed four (~8 kb, ~2.5 kb, ~2 kb, ~1.5 kb) of the four bands seen in the genomic blots (Fig. 8B). These data, together with other mapping studies (data not shown), suggest that MRL3 is a single-copy gene, greater than 15 kb in length. Further studies revealed that integrated HBV DNA is about 8 kb downstream from the 3' end of the MRL3 gene (data not shown). Thus, the HBV enhancer sequence must be greater than 23 kb distant from the

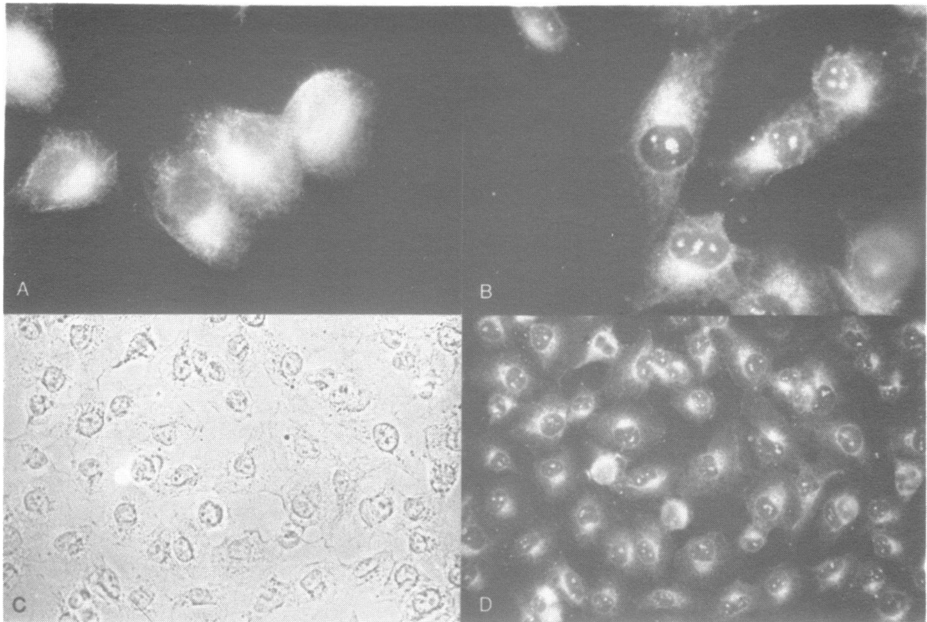

FIGURE 6. Immunochemical localization of the MRL3 protein in Mahlavu hepatoma cells. A, control serum; B & D, rabbit anti-MRL3 protein; C, phase contrast picture of same field as D, showing the fluorescent spots to correspond to nucleoli. See text for experimental procedures.

MRL3 promoter. It seems unlikely that the HBV enhancer can operate over such a distance. Genomic Southern blots of DNA from two HCC specimens revealed no integrated HBV DNA near this gene (data not shown). Thus the integration of HBV DNA at this locus is not a consistent feature of HCC cells. Further, no gross rearrangement of the MRL3 gene sequences is evident in several HCC's as compared to normal human placental DNA (Fig. 6A). Thus, MRL3 does not behave like most cellular oncogenes during an oncogenic transition.

DISCUSSION

We report here the characterization of a gene (MRL3) whose expression is enhanced in fetal and tumor tissues. The central 210 residues of the encoded protein sequence (349 residues) is 30% identical to the 210 residue L3 protein of E. coli ribosomes; the most similar region, 40 residues long, contains more than 50% identical residues. This gene could be the mammalian homologue of the bacterial ribosomal protein L3 gene. The MRL3 gene product is associated with the nucleolus, where nascent ribosomes are assembled, and is also associated

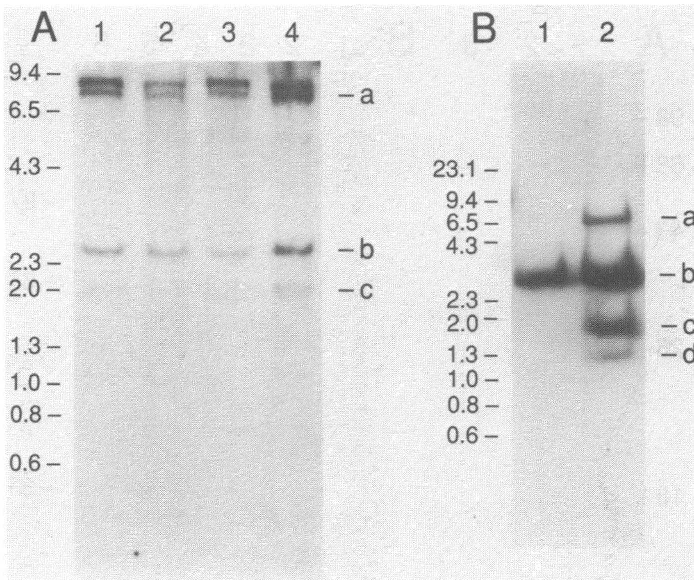

FIGURE 7. A. Immunoprecipitation of MRL3 protein by pXG6 monoclonal antibody. ^{35}S-methionine labeled MRL3 proteins were synthesized by transcription and translation *in vitro* of MRL3 cDNA cloned in a SP6 vector. The labeled protein was then immunoprecipitated by treatment with pXG6 antibody (lane 2) or an irrelevant $IgG2_a$ monoclonal antibody purchased from Sigma (lane 3), followed by rabbit anti-mouse IgG and Pansorbin (19). The precipitate was separated on an SDS acrylamide gel and revealed by autoradiography. The MRL3 protein (apparent molecular mass 38 kd) is specifically precipitated by pXG6. Lane 1 shows total, unprecipitated synthesized protein. B. Western blot localization of MRL3 protein in subcellular fractions. HeLa cells were separated into nuclear (lanes 3 and 6), ribosomal (lanes 2, 5) and post-ribosomal (lanes 1, 4) fractions (31), and the proteins electrophoresed on a SDS acrylamide gel. The proteins were then blotted on nitrocellulose and probed with pXG6 (lanes 1-3) or an irrelevant antibody (lanes 4-6). Bound antibody was visualized by ELISA (22). The MRL3 protein is present in the nuclear and ribosomal fractions but not in the post-ribosomal supernatant. The numbers on the side indicate the molecular mass in kd of protein standards.

with ribosomes. However, the MRL3 protein differs significantly from the bacterial ribosomal protein. It contains a 96 residue amino terminal extension, and a 43 residue carboxy terminal addition rich in proline and acidic residues. Unlike many eukaryotic ribosomal genes which are multicopied (28,29) MRL3 appears to be a single copy gene. Another function for this protein is therefore possible but not necessarily to be expected. For example, the rat S21 protein shows only 57% identity to the corresponding yeast protein, and shows no recognizable sequence similarity to the presumed cognate bacterial protein (27).

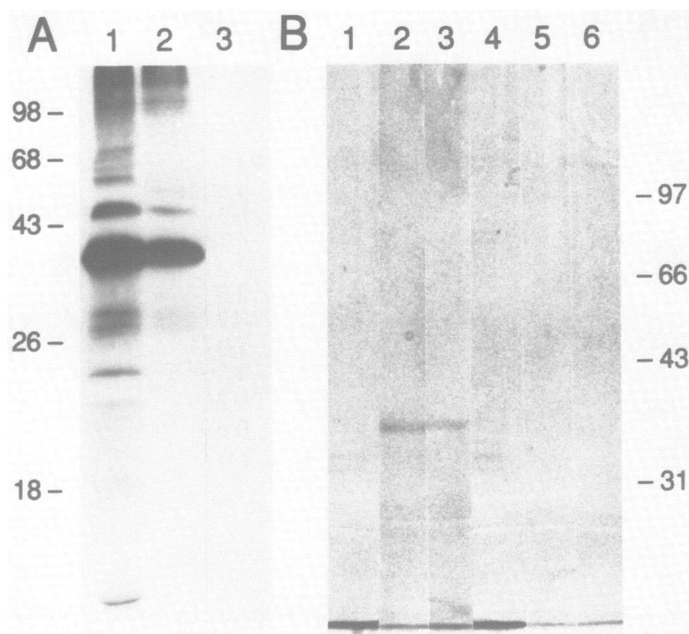

FIGURE 8. Southern blots of the MRL3 gene. A. Genomic blot. 1, normal human DNA isolated from placenta; 2, hepatoma DNA of a patient; 3, Alexander DNA; 4, Mahlavu DNA. Approximately 10 µg of each DNA sample digested by EcoRI was loaded on each lane. No obvious rearrangement of the MRL3 gene in these three hepatoma cells can be detected. B. Phage DNA blot. 1, clone 13 isolated from Alexander genomic library; 2, a phage clone (clone 3c) isolated from a Charon 4A normal human genomic library (26) using the MRL3 cDNA probe. Clone 3c contains an insert approximately 17 kb long. Clone 3c contains the 5' extension of the MRL3 gene and overlaps clone 13 for about 7 kb. 5 µg of the phage DNA digested by EcoRI was loaded on each lane. The blots were hybridized with the MRL3 cDNA probes. a,b,c, show the corresponding DNA fragments in clone 3c and chromosomes, although it is not clear which of the a doublets is present in the phage clone. A longer exposure of gel A reveals the d fragment in (A) (data not shown). Based on these Southern blot results, it seems unlikely that MRL3 gene belongs to a multigene family and the total length of the EcoRI fragments of the genomic Southern is approximately 20-25 kb. The numbers on the side indicate the length in base pairs of DNA standards.

The higher level of MRL3 gene expression in Alexander cells is probably not mediated by the HBV enhancer. Genomic mapping and partial DNA sequencing of isolated genomic clones indicate that the promoter of the MRL3 gene is at least 23 kb from the HBV enhancer. It seems unlikely that the HBV enhancer operates over such extended distances.

Even though MRL3 is highly expressed in other hepatomas studied, the integrated HBV sequences are not proximal to MRL3. Furthermore, MRL3 is

highly expressed in other tumors and in rapidly growing, e.g., fetal, cells. Finally overexpression of MRL3 in an SV40 derived vector in mouse fibroblast (NIH 3T3) cells does not result in cell transformation. We therefore conclude that the MRL3 gene product is not oncogenic and that overexpression is not related to HBV induced oncogenesis. A high level of expression of MRL3 appears to be a general property of actively dividing cells.

The MRL3 protein is thus intrinsically interesting. Relatively few mammalian ribosomal genes have been cloned and little is known regarding the regulation of their gene expression. The functional role of MRL3, presumably in ribosomes, and the regulation of MRL3 expression can now be effectively studied.

ACKNOWLEDGEMENTS

We thank Mark Selby for helpful discussions, Jeffrey Edman for providing the rat liver cDNA library, C.H. Woo for technical assistance, and Leslie Spector for expert assistance in preparing the manuscript. This work was supported by NIH grant AI19744 (to WJR) and grants from FPC, USA and UCSF REAC (to TSBY).

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