Vol. 212, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 6, 1995 Pages 21-26

Differential Display and Cloning of Messenger RNAs from the Late Phase of Rat Liver Regeneration

Siddhartha Kar and Brian I. Carr*

Pittsburgh Transplantation Institute,
University of Pittsburgh School of Medicine, Pittsburgh, PA 15260

Received May 12, 1995

Liver regeneration allows for recovery from hepatic injuries and regeneration after partial hepatectomy has been extensively used as a model system to study mitogenesis. Many genes have been identified that are induced in the early growing phase of liver regeneration but only a few genes have been identified that are induced in the later stages of regeneration before growth arrest. We used the newly discovered differential display method to identify several genes that were found to be induced at this later stage. Two of them were analyzed further. DNA sequencing of one of them revealed perfect homology to ribosomal protein S24. The sequence of the other gene did not have extensive homology to any sequence in the databases. These results may suggest a role of these two genes in the growth arrest phase of liver regeneration.

© 1995 Academic Press, Inc.

In healthy mammals there is a well-defined relationship between body and liver weight (1). The cells of an adult mammalian liver remain normally in the quiescent state, however they have the capacity to regenerate (2, 3). Liver regeneration can be induced by any acute treatment, surgical or chemical, that will remove or destroy a large portion of the liver. Loss of liver parenchyma induces liver cell proliferation which rapidly restores the original liver mass and subsequently cell proliferation stops.

The most commonly used approach for inducing liver regeneration is by performing a two-thirds partial hepatectomy (4). After partial hepatectomy in rat, the majority of the remaining quiescent liver cells reenter cell cycle. The kinetics of the regenerative response have been well described (5, 6). The first round of DNA synthesis starts in the parenchymal cells (hepatocytes) at 12-16 h after hepatectomy and reaches a peak within 22-24 h. DNA synthesis in the nonparenchymal cells (endothelial cells, bile ductule cells, Kupffer cells and Ito cells) reaches a peak at 48 h after hepatectomy. By about 9 days the liver regains its initial mass and the cells become quiescent again.

A complex interplay of positive and negative growth controls is involved in the process of liver regeneration (2, 7, 8). Key factors responsible in the growth control were discovered by

*Corresponding Address: Dr. Brian I. Carr, Pittsburgh Transplantation Institute, E1550 BST, University of Pittsburgh Medical Center, Pittsburgh, PA 15260.

0006-291X/95 $12.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

using hepatocyte cultures in serum-free medium (primary cultures) (9, 10). In order to understand the molecular mechanisms involved in liver regeneration, expression of many genes that were induced during the 9-day regenerative period after partial hepatectomy were analyzed (11). The genes were grouped by their expression pattern into immediate-early, delayed-early and liver specific genes.

Little is known about the mechanisms that terminate liver regeneration. One possibility is that the hepatocytes that enter DNA synthesis during regeneration are programmed to go through only one or two cell divisions and then return to the quiescent state. However several growth inhibitors have been identified suggesting a more active role played by them in stopping liver regeneration. We were interested in identifying candidate genes that might be involved in stopping liver growth after regeneration. We hypothesized that these genes would be induced at a later stage after partial hepatectomy.

Populations of mRNA expressed in rat liver tissue at 0 days and 5 days after partial hepatectomy were compared by the newly discovered arbitrarily primed differential display PCR (AP-PCR) method (12). Candidate mRNAs that were induced at 5 days after partial hepatectomy were purified and partially sequenced. By this method we identified two genes, one of which (clone 2-6) had perfect homology with ribosomal protein S24. Sequence of the other clone (2-4) did not have significant homology to any sequence in the databases.

MATERIALS AND METHODS

Rat liver tissue preparation: Male Fischer rats were chosen for partial hepatectomy. The surgical operation was done following a previously described method (4).Whole livers were perfused with 20 ml of saline and were harvested at 0 and 5 days after partial hepatectomy. The harvested livers were quickly frozen in liquid nitrogen and stored at -70°C.

RNA extraction and purification from liver tissue: Total RNA was extracted from liver tissues using the TRIZOL reagent kit (Life Technologies, Gaithersberg, MD) based on the method first described by Chomczynski and Sacchi (13). Contaminating traces of DNA was eliminated from the total RNA sample (50 µg) by digestion at 37°C for 30 min with DNase I (10 u) using the MessageClean kit (GenHunter Corp., Brookline, MA).

AP-PCR comparison of RNA samples: AP-PCR was performed on the purified total RNA samples using the RNAmap kit (GenHunter Corp., Brookline, MA). Briefly, cDNAs were reverse transcribed from the total RNA using 4 different anchor primers containing oligo dT. The cDNAs were then amplified by sets of anchor and arbitrary primers in presence of ^{35}S-dATP. The radioactively labeled PCR fragments were resolved on a 6% DNA-sequencing gel. Fragments amplified from RNAs of liver tissue at 0 and 5 days after partial hepatectomy were compared on adjacent lanes of the gel. The gel was dried without fixing and autoradiographed for 2 days on Kodak XAR-5 film. The dried gel was marked asymmetrically with radioactive ink for future alignment with the exposed film.

Purification of cDNA probes: The exposed film was aligned with the dried gel and the candidate PCR-fragments, which were seen to be differentially expressed, were cut out of the gel. DNA was released from the gel slice by soaking it in water for 10 min and then boiling in water for 15 min. The released DNA was ethanol precipitated and reamplified by PCR with the original set of primers. The amplified DNA was cloned directly into a plasmid vector by the TA cloning kit (Invitrogen, San Diego, CA).

Northern blot analysis: Northern blot analysis was performed using standard method (7). 30 µg of total RNA from different liver tissues were denatured and run on a 1% agarose gel counting glyoxal. The resolved RNAs were transferred to charged nylon membranes (Whatman, Hillsboro, OR) and the blots were probed with radioactively labeled probes.

Generation of radiactively labeled probes: ^{32}P-labeled probes were generated from the cloned candidate PCR-fragments by PCR amplification with the appropriate set of primers in presence

of ^{32}P-dCTP. The probe was purified on a mini spin-column containing Biogel P-30 (Bio Rad Laboratories, Richmond, CA).

DNA sequencing and sequence analysis: DNA sequence of the cloned fragments were determined by the dideoxy method using the sequenase kit (USB, Cleveland, OH). Sequence comparison with databases and other sequence analysis were done at the computing facility of Pittsburgh Supercomputing Center.

RESULTS

Differential display with AP-PCR: AP-PCR was performed on RNA from liver tissues at 0 and 5 days after partial hepatectomy. The PCR fragments were run side-by-side on five DNA sequencing gels. A total of 80 PCR reactions were done with 4 anchor primers and 20 different arbitrary primers for each RNA sample. Several PCR fragments were seen to be either induced or suppressed at 5 days after partial hepatectomy when they were compared to 0 days (Figure 1).

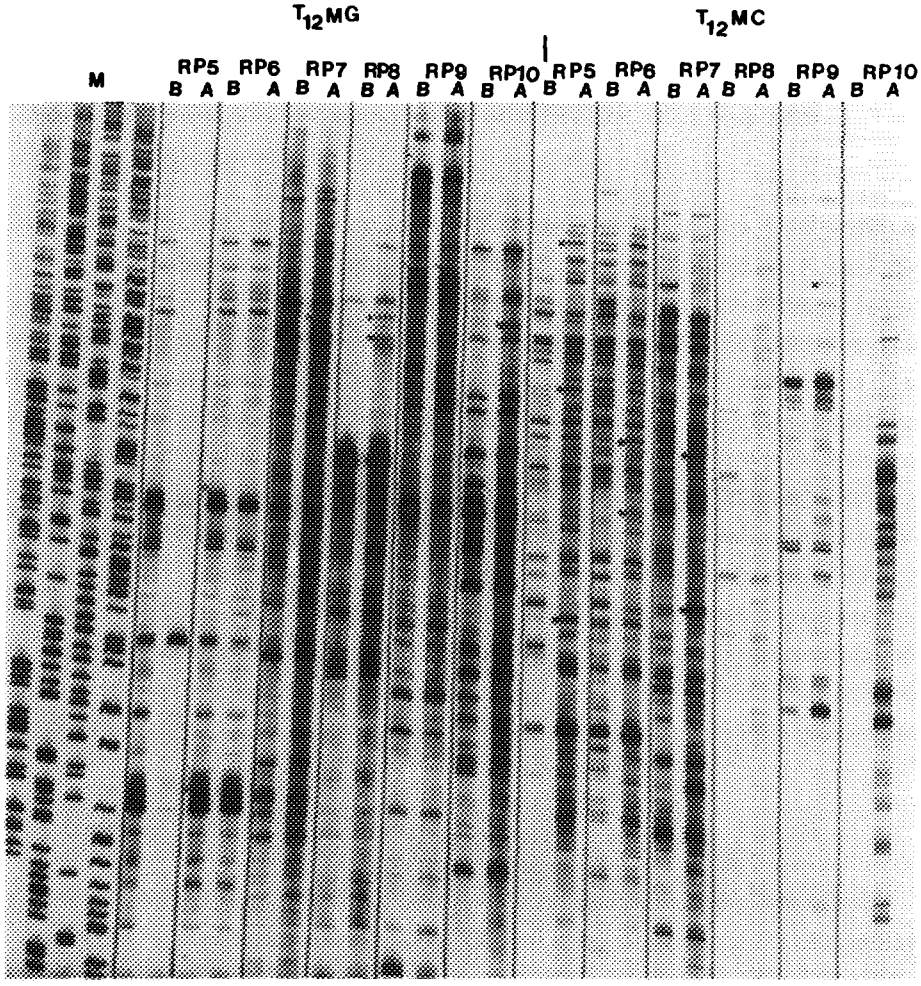

Figure 1. Differential display gel. A representative gel showing differential display of mRNAs from rat liver at day=0 (lane B) and day=5 (lane A) after partial hepatectomy. Anchor primers $T_{12}MG$ and $T_{12}MC$ and arbitrary primers RP5-RP10 were used. Lane M shows size standards. Several induced bands are marked with dots.

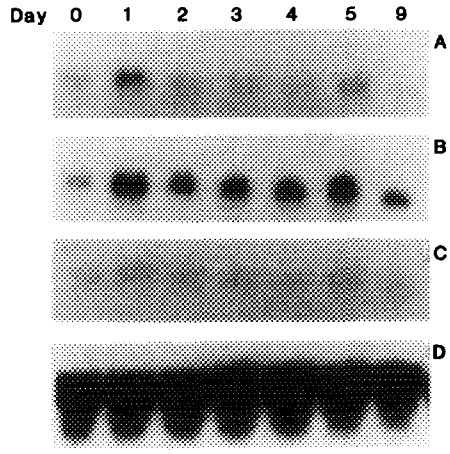

Figure 2. mRNA expression of clones 2-4 and 2-6 in rat liver after partial hepatectomy. Rat livers were harvested at days 0, 1, 2, 3, 4, 5 and 9 after 70% partial hepatectomy or sham surgery. Northern blots containing the liver RNAs were probed with clones 2-4, 2-6 and 18S rRNA control. Panels A and B: liver RNA after partial hepatectomy probed with clones 2-4 and 2-6. Panel C: liver RNA after sham surgery probed with either clone 2-4 or 2-6. Panel D: 18 S rRNA control for partial hepatectomy samples.

Fifteen differentially expressed fragments were excised from the gels and were reamplified and purified. Two of the fragments (clones 2-4 and 2-6) whose expression were induced at 5 days after partial hepatectomy were cloned and further characterized.

Northern analysis with the probes: RNAs were extracted from liver tissue at different times (0 to 9 days) after partial hepatectomy or sham operation. The RNAs were denatured, separated by electrophoresis on an agarose gel and subsequently blotted onto charged nylon membranes. The northern blots were probed with radioactively labeled clones 2-4 and 2-6 (Figure 2). Both the probes hybridized to mRNA species of about 0.5 kb and their expression had two peaks at 1 day and 5 days after partial hepatectomy. The control northern blots with RNAs from livers after sham operation did not show any change in the expression of these clones.

DNA sequencing of clones 2-4 and 2-6: DNA sequence of clones 2-4 and 2-6 were determined by dideoxy method (Figure 3). DNA sequence of clone 2-6 was found to have a perfect homology to ribosomal protein S-24. DNA sequence of clone 2-4 did not show significant homology to any sequence in the Genbank/EMBL databases.

DISCUSSION

Most of the genes, that had been identified to change their expression after partial hepatectomy of rat liver, were found to be induced shortly after hepatectomy. Whether some of these genes also play a role at the later stages of growth arrest of the regenerating liver is not clear. Only one gene had been reported so far that was induced 2 days after partial hepatectomy and with an elevated expression thereafter. This was identified as C/EBPα, a tissue-specific elongation factor (11).

Figure 3. DNA sequence of clones 2-4 and 2-6. (A) Sequence comparison of clone 2-6 with ribosomal protein S24. (B) sequence of clone 2-4. The flanking differential display PCR primers are underlined and putative polyadenylation sites are written in lower case letters.

Our search for genes that were induced at 5 days after partial hepatectomy identified many induced genes. Two of them, 2-4 and 2-6, have been cloned and sequenced. The genes corresponding to these clones were also found to be induced both at day 1 and day 5 after partial hepatectomy. Both the clones have authentic polyadenylation sites located within 30 bp upstream from the poly A tail indicating that they are the 3' end of authentic mRNAs. Two mismatches between the 5' end sequence of the arbitrary primer (AGCCAGCGAA) and the original target cDNA (AAACAGCGAA) of clone 2-6 were found which is probably due to some degree of degeneracy that can be exhibited by the arbitrary primers.

DNA sequence of clone 2-6 showed perfect homology to ribosomal protein S24. Human ribosomal phosphoprotein expression has recently generated interest because of their increased expression in specific primary and metastatic tumors (14, 15). Some other ribosomal proteins including S6 and S27-ubiquitin hybrid had been reported to have increased expression in human colorectal cancer (16, 17). In general, an increase in ribosomal protein gene expression correlate with proliferation of tumor cells. However, some ribosomal proteins were not found to be induced during cell proliferation (16) and S24 might have a dual role both in cell proliferation and growth arrest. DNA sequence of clone 2-4 was not found to have homology to any known sequence in the databases. The lack of homology for clone 2-4 with known sequences does not eliminate the possibility that it may be homologous to known proteins, since non-coding 3'

region of mRNA shows great species to species variation. 2-4 also showed two peaks of induction, one at the early and one at the late stage of regeneration. Like clone 2-6 it may also have a dual role both in the early and late stages of liver regeneration.

This newly discovered differential display technique proved to be a relatively easy method to identify differentially expressed genes during liver regeneration when it is compared to the previously described technically difficult method of subtractive library construction (11). To our knowledge, this is the first use of this new technique for studying gene expression during liver regeneration. Identification of these genes and their significance in the regenerative process remains to be determined.

REFERENCES

1. Kam, I., Lynch, S., Svanas, G. et.al. (1987) Hepatology 7. 362-366.
2. Michalopoulos, G. (1990) FASEB J. 4. 176-187.
3. Fausto, N., and Mead, J.E. (1989) Lab. Invest. 60. 4-13.
4. Higgins, G.M., and Anderson, R.M. (1931) Arch. Pathol. 12. 186-202.
5. Grisham, J.W. (1962) Cancer Res. 22. 842-849.
6. Rabes, H.M., Wirshing, R., Tuczek, H.V., and Iseler, G. (1976) Cell Tissue Kinet. 6. 517-532.
7. Hodgeson, H.J.F. (1993) J. Royal Coll. Physicians London 27. 278-283.
8. Francavilla, A., Polimeno, L., Barone, M, Azzarone, A., and Starzl, T.E. (1993) J. Surg. Oncology Supplement 3. 1-7.
9. Seglen, P.O. (1976) Methods Cell Biol. 13. 29-83.
10. Hasegawa, K., Kar, S., and Carr, B.I. (1994) J. Cell. Physiol. 158. 215-222.
11. Haber, B.A., Mohn, K.L., Diamond, R.H., and Taub, R. (1993) J. Clin. Invest. 91. 1319-1326.
12. Liang, P., and Pardee, A. (1992) Science 257. 967-971.
13. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162. 156-159.
14. Elvin, P., Kerr, I., McArdle, C., and Birnie, G. (1988) Br. J. Cancer 57. 36-42.
15. Sharp, M., Adams, S., Elvin, P., Walker, R., Brammar, W. and Varley, J. (1990) Br. J. Cancer 61. 83-88.
16. Mafune, K., Wong, J., Staniunas, R., Lu, M., Ravikumar, T, Chen, L., and Steele, G. Jr. (1991) Arch. Surg. 126. 462-466.
17. Staniunas, R., Mafune, K., Lu, M., Chen, L., and Steele, G. Jr. (1990) Surg. Forum 41. 457-459.