Vol. 124, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1984 Pages 164-171

MOLECULAR CLONING AND CHARACTERIZATION OF A cDNA CLONE FOR A PROTEIN SPECIFICALLY EXPRESSED IN EMBRYO AS WELL AS IN A CHEMICALLY INDUCED PANCREATIC B CELL TUMOR OF RAT

Gen-Ichiro Soma,* Namiko Kitahara,and Toshiwo Andoh

Department of Hygienic Chemistry, Meiji College of Pharmacy,
1-22-1 Yato-cho, Tanashi, Tokyo 188, Japan

Received August 30, 1984

cDNAs were molecularly cloned for proteins specifically expressed in embryo as well as in a chemically induced rat pancreatic B cell tumor in which virally related oncogenes such as v-myc, v-src, v-yes, v-mos and v-kis were previously demonstrated not to be expressed. A plasmid cDNA library consisting of 48,000 independent colonies was constructed from poly(A) containing cytoplasmic RNA isolated from 12 day rat embryo. The library was screened by hybridization with ^{32}P-labelled cDNA synthesized from poly(A) containing RNA of rat pancreatic B cell tumor or normal islet B cells. Two clones were obtained which showed a clearly positive reaction only with tumor probe. Nucleotide sequence of one of them harboring insert of 615 nucleotides was determined and its amino acid sequence of 119 residues was deduced, which showed that the protein encoded by this mRNA is highly basic, basic residues/acidic residues being 1.63.

The wide variety of enzymatic and other biochemical differences between transformed and normal cells (1,2) as well as antigenic differences (3) demonstrate that complex alterations in gene expression accompany tranformation. Thus, although extensive homology has been found between the mRNA populations of normal human fibroblasts and their SV40-transformed counterparts (4), hamster embryo fibroblasts and the same cells transformed with bezopyrene (5) and rat liver and a minimun-deviation hepatoma cell line (6), Groudine and Weintraub (7), however, have reported recently that activation of host cell genes by transforming viruses may be more extensive, involving the appearance of perhaps 1,000 new transcripts. Augenlicht and Kobrin (8) have succeeded in cloning of sequences specifically expressed in a mouse colon tumors.

*To whom all correspondence and reprint requests should be addressed.

Abbreviations used: cpm, counts per minute; SSC, standard saline citrate, 0.15 M NaCl, 0.015 M sodium citrate.

0006-291X/84 $1.50

Furthermore, in various forms of malignancies some of the genes which are expressed during embryonal development such as α-fetoprotein (9) in fetal liver and carcinoembryonic antigen (10) in gastrointestinal tracts are expressed in transformed cells (2). Cellular genes responsible for malignant transformation of the cells (c-onc) have recently been isolated and extensively analyzed, and were revealed to be cellular homologues of viral oncogenes (v-onc) (11-17). Transcription of some of these c-onc genes has been detected in normal vertebrate cells (18-22), especially during prenatal development (23, 24); some of the c-onc gene products have recently been found to be closely related to normal cellular growth factors (25), *e.g.* sis and erb B being part of PDGF, platelet derived growth factor, and EGF, epidermal growth factor, respectively. In order to elucidate the role of such oncodevelopmental expression of genes in general we attempted to search in rat embryo for the genes specifically expressed in tumors as well as in embryos but not in corresponding normal differentiated cells. To this end we employed chemically induced rat pancreatic B cell tumor and corresponding normal B cells. In the present paper we describe for the first time isolation of two such clones from rat embryo which met these criteria.

MATERIALS AND METHODS

Northern and Southern Blot Hybridization: Pregnant Wistar rats were purchased from Tokyo Jikken Dohbutsu Co., Ltd., Tokyo. At day 12 of prenatal development embryos were isolated and polyadenylated (poly(A)$^+$) RNA was prepared as described previously (26). Similarly poly(A)$^+$ RNAs were isolated from rat Langerhans islet B cell tumor induced by streptozotocin and nicotinamide (27-29) and normal adult islets which consist mainly of B cells (29). ^{32}P-labelled cDNA probes were synthesized as described previously (26) by incubation at 42°C for 90 min in 20 µl of reaction mixture containing 50 mM Tris-HCl, pH 8.3; 30 mM NaCl; 10 mM $MgCl_2$; 0.5 mM dithiothreitol; 4 mM Na-pyrophosphate; dATP, dGTP and dTTP at 1 mM each; 10 µCi of [α-^{32}P]dCTP (410 Ci/mmol, Radiochemical Center, Amersham); 1 unit of avian myeloblastosis virus reverse transcriptase (EC 2.7.7.49) purchased from Seikagaku Kogyo, Inc.; 50 ng poly(A)$^+$ RNA. The specific activity was about 1 x 10^8 cpm/µg. Electrophoresis and hybridization of poly(A)$^+$ RNA with ^{32}P-labelled probe were carried out as described by Thomas (30): Poly(A)$^+$ RNA dissolved in water were glyoxalated in 1 M glyoxal in 50 % dimethylsulfoxide containing 10 mM phosphate buffer, pH 7.0 for 1 hr at 50°C, and 100 ng each of the RNA was subjected to electrophoresis for 3 hr at room temperature on 1.2 % agarose gel in 10 mM phosphate buffer, pH 7.0. After electrophoresis RNA was transfered to nitrocellulose paper. After baking for 1 hr at 80°C the blot was prehybridized for at least 4 hr at 42°C in hybridization buffer containing 5 x SSC (Standard Saline Citrate); 50 mM phosphate buffer, pH 6.5; 1.0 x Denhard's solution; 250 µg/ml of sonicated

and denatured calf thymus DNA in 50 % formamide. Subsequently the blot was hybridized for 18 hr at 42°C with 5 x 10^5 cpm of ^{32}P-cDNA probe per ml of hybridization buffer except that the concentrations of all components were decreased to 0.8 x and contained 10 % dextran sulfate. After hybridization the blot was washed three times in 2 x SSC at 42°C for 0.5 hr and exposed to X-ray films (Kodak X-AR) with intensifying screen at -80°C. Plasmid DNAs isolated from cDNA clones of *E. coli* and one µg each of the DNA was electrophoresed and transfered to nitrocellulose paper as described by Southern (31). Blots were hybridized with ^{32}P-cDNA probes prepared from normal islet B cells or B cell tumor.

Preparation of cDNA Clones: Construction of cDNA library from rat embryo was carried out according to the method of Goodman and MacDonald (32) with some modifications (to be described elsewhere). Five µg of poly(A)$^+$ RNA from embryo was used for the preparation of cDNA. By ligating 240 ng of double-stranded d(C)-tailed cDNA into d(G)-tailed *Pst*I site of pBR322 DNA and transfecting *E. coli* with the recombinant molecules we obtained 4.8 x 10^4 tetracyclin resistant and ampicillin sensitive transformants. The transformants were colony-hybridized (33) with ^{32}P-cDNA probes prepared as described above.

Nucleotide Sequence Analysis: Nucleotide sequence was determined by the chemical degradation method of Maxam and Gilbert (34). Restriction enzyme fragments were labelled at the 3' end with calf thymus terminal deoxynucleotidyl transferase and [α-^{32}P]dideoxyATP (500 Ci/mmol, Radiochemical Center, Amersham) or labelled at the 5' end with T4 polynucleotide kinase and [γ-^{32}P]ATP (1,000 Ci/mmol, Radiochemical Center, Amersham). Single end-labelled DNAs were prepared for sequence analysis by secondary cleavage with another restriction endonuclease.

RESULTS AND DISCUSSION

Poly(A)$^+$ RNAs were isolated from rat pancreatic B cell tumor induced by streptozotocin and nicotinamide and from isolated pancreatic islets which consist mainly of B cells. After denaturation by glyoxalation these RNAs were electrophoresed on an agarose gel, transfered to nitrocellulose filter and hybridized with ^{32}P-cDNA probe prepared from poly(A)$^+$ RNA from embryos. As shown in Fig. 1, a transcript with 0.8 kilobases (kb) was specifically detected in tumor cell poly(A)$^+$ RNA preparation. Under a longer exposure of the filter, however, we could detect another transcript with slightly less bases only in normal cell poly(A)$^+$ RNA population (data not shown). For further confirmation of these results we carried out a reciprocal experiment in which filter blotted embryonic poly(A)$^+$ RNA was hybridized with ^{32}P-cDNA synthesized from poly(A)$^+$ RNA from either tumor or normal cells. Figure 2 clearly shows that there are two prominent RNA species among embryonic poly(A)$^+$ RNA populations, one having the Mr of 0.8 kb and hybridizing only with tumor probe and the other with slightly smaller Mr and hybridizing only with normal probe, confirm-

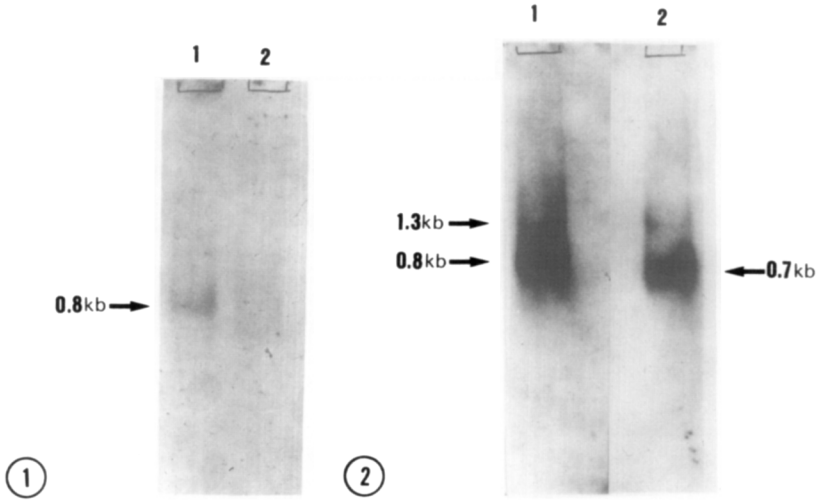

Figure 1. Tumor-specific expression of genes which are also expressed in embryos. Poly(A)$^+$ RNAs were isolated from rat Langerhans islet B cell tumor, normal adult islets and embryos at day 12 of prenatal development. Poly(A)$^+$ RNAs from normal islets and islet B cell tumor were glyoxalated and 100 ng each of the RNA was electrophoresed on agarose gel and transfered to nitrocellulose filter. The filter was hybridized with ^{32}P-cDNA probe synthesized from poly(A)$^+$ RNA of embryos, and autoradiographed. Lane 1, B cell tumor poly(A)$^+$ RNA; lane 2, normal islet poly(A)$^+$ RNA. The arrow indicates the position of RNA with approximately 800 bases which was determined using glyoxalated *Hae*III digests of ϕX174 replicative form DNA.

Figure 2. Expression in embryos of genes which are specifically expressed in B cell tumor. Poly(A)$^+$ RNA from embryos was electrophoresed, transfered to nitrocellulose filter, and hybridized with ^{32}P-cDNA prepared from B cell tumor or normal islet poly(A)$^+$ RNA, as described in Figure 1 and Materials and Methods. Lane 1, embryo poly(A)$^+$ RNA hybridized with tumor ^{32}P-cDNA; lane 2, embryo poly (A)$^+$ RNA hybridized with normal B cell ^{32}P-cDNA.

ing the result of Figure 1. In addition we detected a third RNA species which possesses Mr of about 1.3 kb and hybridizes with both probes.

These results strongly suggest that there are such genes in rat genome that are specifically expressed in embryos as well as in B cell tumors but not in corresponding normal B cells. It is obviously of great importance to elucidate the function of these genes in embryonal development as well as in tumor cells. To this end we attempted to isolate them from a plasmid cDNA library of 48,000 tranformants constructed from messenger RNA of 12 day embryos. One thousand transformants were randomly selected and colony-hybridized with ^{32}P-cDNA probes synthesized from embryo, B cell tumor and normal B cell poly(A)$^+$ RNAs. We found that all of the transformants selected were positive with embryo probe, indicating that they possessed cDNA inserts, and 150 out of 1,000

were positive with B cell tumor probe. These 150 clones were rescreened with normal and tumor probes. Forty candidate clones positive with tumor probe only were selected and plasmid DNAs were prepared therefrom, and subjected to Southern blot analysis using tumor and normal probes. Finally two plasmid DNAs were found which gave clearly positive reaction with only tumor probe (data not shown). One clone denoted as pR_{ET2} harboring longer insert of the two has been subjected to further analyses. Northern blot analysis was carried out using poly(A)$^+$ RNA from embryo: the RNA was electrophoresed and blotted to nitrocellulose filter and hybridized with ^{32}P-nick-translated cDNA insert of the plasmid pR_{ET2}. Figure 3 shows that the mRNA corresponding to the cDNA insert of the plasmid was approximately 800 bases long. In order to confirm the specific expression of the mRNA in B cell tumor poly(A)$^+$ RNAs were similarly blotted and probed with the ^{32}P-cDNA insert of the plasmid.

Results shown in Fig. 4 clearly demonstrated that the pR_{ET2} mRNA with about 800 bases was detected only in B cell tumor RNA. These findings unequivocally demonstrated that we have cloned one of the mRNAs specifically expressed in both embryo and B cell tumor of rat, and thus the cDNA clone pR_{ET2} being one of the oncodevelopmentally expressed genes. What role the pR_{ET2} gene encoded protein plays remains to be elucidated. However, it may possibly play a key function in embryogenesis as well as in carcinogenesis. Müller *et al.* (23) revealed that some oncogenes such as fos and abl were expressed during embryonal development but not in normal tissues. This result strongly suggested that those genes intimately associated with carcinogenesis are also required for embryonal development. Preliminary analysis revealed that in B cell tumor some virally related oncogenes such as v-myc, v-src, v-yes, v-mos and v-kis were not expressed, and further that the cDNA clone pR_{ET2} did not hybridize with the above v-oncogenes. In order to get further insight into the function of the clone pR_{ET2} it is of great interest to know the amino acid composition and sequence of the putative product of the gene and to compare its sequence with those of oncodevelopmentally expressed genes such as oncogene products, α-fetoprotein or carcinoembryonic antigens. For this pur-

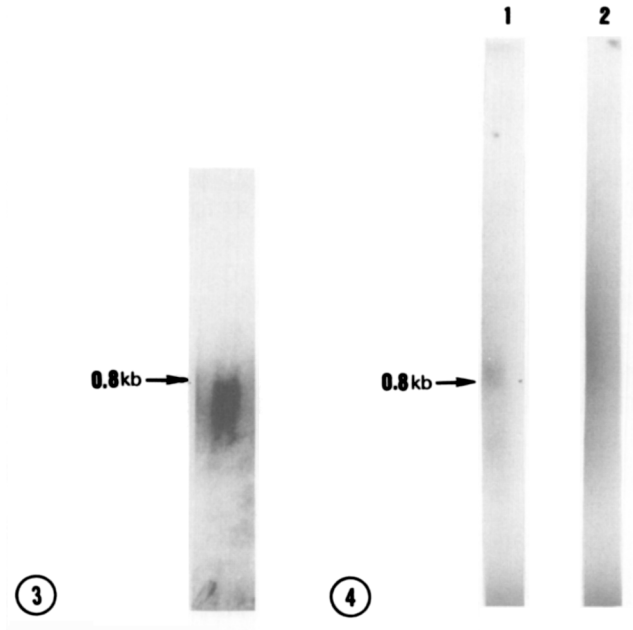

Figure 3. Northern blot hybridization to detect in embryo the mRNA corresponding to the clone pR_{ET2}. Hundred ng of poly(A)$^+$ RNA from rat embryo was glyoxalated and electrophoresed on agarose gel followed by transfer to nitrocellulose filter. The filter was hybridized with ^{32}P-nick-translated insert DNA of pR_{ET2}. The size estimation was done as described in Figure 1.

Figure 4. Expression of pR_{ET2} gene in B cell tumor of rat. Fifty ng of poly (A)$^+$RNAs from B cell tumor (lane 1) and normal B cells (lane 2) were glyoxalated and electrophoresed on agarose gel followed by transfer to nitrocellulose filter. The filter was hybridized with ^{32}P-nick-translated insert DNA of pR_{ET2}.

poses we determined the nucleotide sequence of the cDNA insert of the clone pR_{ET2}. Figure 5 shows the whole sequence of 615 nucleotide insert excluding poly(A) tract and poly(dG)·(dC) tails. Although the cDNA insert covers almost entire region of pR_{ET2} mRNA, *i.e.* 615 nucleotides plus poly(A) tract appraoching 800 nucleotides estimated for pR_{ET2} mRNA (see Figs. 2 and 3), no possible initiation codon ATG was found in the 5' upstream region. Therefore, amino acid alignment was temporarily determined according to the longest possible open reading frame spanning nucleotide number 1 through 360. If this alignment was correct, the protein deduced is highly basic, *i.e.* 26 residues of lysine plus arginine vs 16 residues of glutamic acid plus aspartic acid, and resembles histone H2B in amino acid composition. When the nucleotide sequence was compared with those of known oncogenes, there was no homology between them,

Figure 5. Nucleotide sequence of pR_{ET2} cDNA insert and its amino acid sequence. Nucleotide residues are numbered in 5' to 3' direction begining with the residues of TCT triplet encoding the predicted first amino acid serine of the putative protein. The predicted amino acid sequence is displayed above the nucleotide sequence.

suggesting that the cloned genes are distinct from those oncogenes known to induce a wide spectrum of malignancies including sarcomas, carcinomas, lymphomas and leukemias.

It has been widely accepted that change in the pattern of gene expression and hence alteration of the constitution of various enzymes including isozymes ensues as a consequence of malignant transformation of cells (1-6). Our results lend support to this contention and establish a general method of isolating genes which should play some important roles in prenatal development as well as in malignant transformation of cells. We should like to refer to such genes as "oncodevelopmental genes". Although possibility still remains that the cloned genes are hitherto uncharacterized oncogenes, they could more likely be among those genes involved in prenatal development as well as in the manifestation of various properties of tumor cells.

REFERENCES

1. Strand, M. and August, J. T. (1977) Proc. Nat. Acad. Sci. USA 74, 2729-2733.
2. Ibsen, K. H. and Fishman, W. H. (1979) Biochim. Biophys. Acta 560, 243-280.
3. Old, L. (1981) Cancer Res. 41, 361-375.
4. Willians, J. G., Hoffman, R. and Perman, S. (1977) Cell 11, 901-907.
5. Moyzis, R. K., Grady, D. L., Li, D. W., Mirvis, S. E. and Ts'o, P. (1980) Biochemistry 19, 821-832.
6. Jacobs, H. and Birnie, G. D. (1980) Nucleic Acids Res. 8, 3087-3103.
7. Groudine, M. and Weintraub, H. (1980) Proc. Nat. Acad. Sci. USA 77, 5351-5355.
8. Augenlicht, L. H. and Kobrin, D. (1982) Cancer Res. 42, 1088-1093.
9. Sell, S., Becker, E. F., Leffert, H. L. and Watabe, H. (1976) Cancer Res. 36, 4239-4249.
10. Gold, P. and Freedman, S. O. (1965) J. Exp. Med. 122, 467-481.
11. Shih, C., Shilo, B., Goldfarb, M., Dannenberg, A. and Weinberg, R. (1979) Proc. Nat. Acad. Sci. USA 76, 5714-5718.
12. Cooper, G., Okenquist, S. and Silverman, L. (1980) Nature 284, 418-421.
13. Murray, M., Shilo, B., Shih, C., Cowing, D. and Weinberg, R. (1981) Cell 25, 355-361.
14. Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J. and Wigler, M. (1981) Cell 27, 467-476.
15. Eva, A., Tronick, S. R., Gol, R. A., Pierce, J. H. and Aaronson, S. A. (1983) Proc. Nat. Acad. Sci. USA 80, 4926-4930.
16. Taparowsky, R., Shimizu, K., Goldfarb, M. and Wigler, M. (1983) Cell 34, 581-586.
17. Souyri, M. and Fleissner, E. (1983) Proc. Nat. Acad. Sci. USA 80, 6676-6679.
18. Bishop, J. M. and Varmus, H. E. (1982) In: RNA Tumor Viruses, The Molecular Biology of Tumor Viruses Part III (Weiss, A., Teich, N., Varmus, H. and Coffin, J. eds.) pp. 999-1108.
19. Chen, J. H. (1980) J. Virol. 36, 162-170.
20. Langbeheim, H., Shih, T. Y. and Scolnick, E. M. (1980) Virol. 106, 292-300.
21. Shibuya, M., Hanafusa, H. and Balsuzzi, P. L. (1982) J. Virol. 42, 143-152.
22. Gonda, T. J., Sheiness, D. K. and Bishop, J. M. (1982) Mol. Cell. Biol. 2, 617-624.
23. Müller, R., Slamon, D. J., Tremblay, J. M., Cline, M. J. and Verma, I. M. (1982) Nature 299, 640-644.
24. Müller, R., Verma, I. M. and Adamson, E. D. (1983) EMBO J. 2, 679-684.
25. Heldin, C.-H. and Westermark, B. (1984) Cell 37, 9-20.
26. Soma, G.-I., Obinata, M. and Ikawa, Y. (1980) Biochemistry 19. 3967-3973.
27. Rakieten, M., Gorden, B. S., Beaty, A., Cooney, D. A., Davis, R. D. and Shein, P. S. (1971) Proc. Soc. Exp. Biol. Med. 137, 280-283.
28. Itoh, N., Nose, K. and Okamoto, H. (1979) Eur. J. Biochem. 97, 1-9.
29. Okamoto, H. (1981) Mol. Cell. Biochem. 37, 43-61.
30. Thomas, P. S. (1980) Proc. Nat. Acad. Sci. USA 77, 5201-5205.
31. Southern, E. M. (1975) J. Mol. Biol. 98, 503-517.
32. Goodman, H. M. and MacDonald, R. J. (1980) Meth. Enzym. 68, 75-90.
33. Grunstein, M. and Hogness, D. S. (1975) Proc. Nat. Acad. Sci. USA 72, 3961-3965.
34. Maxam, A. M. and Gilbert, W. (1980) Meth. Enzym. 65, 499-560.