Cloning of sequences expressed specifically in tumors of rat
(recombinant DNA/RNA blot hybridization/ascites hepatomas/middle-repetitive sequence)

Mikio Yamamoto, Yoshiihiko Maehara, Keikichi Takahashi, and Hideya Endo
Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

Communicated by Van R. Potter, August 25, 1983

ABSTRACT The sequences specifically transcribed in tumor cells are believed to be closely related to transformed phenotypes. For the isolation of such sequences, a cDNA clone library was constructed by using poly(A)⁺ RNAs from azo-dye-induced rat ascites hepatomas. Thirty-one tumor RNA-responsive clones were isolated by screening 4,000 clones of this library with conventional techniques, differential colony hybridization, and RNA blot hybridization. These clones were categorized into two groups with respect to their size distribution of mRNAs from which clones were derived. The first group was complementary to a single distinct species, either about 1.5 or 0.6 kilobases in length, of poly(A)⁺ RNA, and the second showed no distinct bands but a smear on a RNA blot. Semiquantitative RNA dot blot assays revealed that the sequences of these clones were expressed very little, if at all, in normal and regenerating livers, while generally high in ascites hepatomas. This specificity was also true for other solid lines of tumors, such as Morris hepatoma 5123D of Buffalo rat and Walker 256 carcinosarcoma of Wistar rat. The smear class sequences were transcribed from middle-repetitive sequences of DNA, indicating that a class of middle-repetitive sequences is specifically transcribed in tumor cells.

Upon cellular transformation, a wide variety of changes occur in morphological and biochemical properties, indicating complex alterations in gene expression. RNA-DNA hybridization analyses have, however, shown extensive homology between mRNA populations of normal cells and transformed counterparts (1–5), although the changes in the individual mRNA levels for enzymes concerned, such as in retinodifferentiation, were remarkable. Grouding and Weintraub (6) have emphasized that many of the mRNAs transcribed in transformed mammalian cells are homologous to mammalian mRNAs transcribed in normal human fibroblasts, while Williams et al. (1) have stressed the overall similarity in host gene expression in normal human fibroblasts compared with their simian virus 40-transformed counterparts. These types of experiments have, however, provided minimal information about the change of the individual mRNA species incident to cellular transformation. mRNA species that are specific or present in abundance in tumor cells are considered to be closely related to the transformed state, especially if the mRNA species having these properties appear commonly in a variety of cancers. It should be useful to isolate such mRNA sequences for the elucidation of the mechanism of transformation in molecular terms. The AH lines of hepatoma appear to be ideal for such study, because a large number of independent lines have been established and serially transplanted for many years (7).

We describe in this report a cloning of sequences expressed abundantly in a variety of lines of chemical-induced rat ascites hepatomas but appearing at a minimal level, if any, in normal liver.

MATERIALS AND METHODS

Tissues. Ascites hepatoma lines AH44, AH60c, AH66, AH109A, AH130, and AH141 are transplantaable rat carcinomas independently induced by p-dimethylaminobenzene. AH66F is a derivative of AH66. These lines have a variety of phenotypes in survival time, transplantability, tumor island formation, metastasis, histological types, etc., though they all have originated from hepatic tumors (7). Yoshida sarcoma of Donryu rat is an ascites tumor of unknown origin. These lines were obtained from H. Sato (Sasaki Institute, Tokyo) and maintained by serial intraperitoneal transplantation in young adult male Donryu rats. Walker 256 carcinosarcoma of Wistar rat and Morris hepatoma 5123D of Buffalo rat were obtained from Kureha Institute (Tokyo) and M. Watanabe (Tohoku University), respectively. Normal livers were obtained from animals of the same strain not bearing tumors. For the preparation of regenerating liver, rats were partially hepatectomized by the method of Higgin and Anderson (8) and liver was excised 12 hr after the operation. Fetal liver was obtained from embryos of 17 days of gestation. All animals were kept in a temperature-controlled room with alternating 12-hr dark–light cycles.

Isolation of Poly(A)⁺ RNA. Tissue was ground in 6 vol of 8 M guanidine-HCl in 20 mM sodium acetate, pH 5.5/1 mM di-thiothreitol in a Waring blender. The homogenate was then overlaid on two layers of cushion composed of 5.7 M CsCl in 100 mM disodium ethylenediaminetetraacetate, pH 7.2 (9)/8 M guanidine-HCl in 20 mM sodium acetate, pH 5.5/1 mM di-thiothreitol and centrifuged for 20 hr at 80,000 × g at 20°C. Precipitated total RNA was washed once with ethanol and then subjected to two cycles of oligo(dT)-cellulose column chromatography to concentrate poly(A)⁺ RNA (10).

Preparation of cDNA Clone Library and Screening. cDNA clones were prepared independently from six lines of hepatoma poly(A)⁺ RNAs according to the method described by Land et al. (11). Insertional inactivation of the β-lactamase gene of the plasmid pBR322 was utilized for the selection of recombinant plasmid clones. The library of about 5,000 cDNA clones was composed of 2,000 clones derived from AH60c and 300–1,000 clones from each of the other five AH lines. Duplicate nitrocellulose filters were prepared, on which cDNA clones were streaked. One filter was hybridized with 32P-labeled cDNA prepared by reverse transcription of normal liver poly(A)⁺ RNA and the other filter was hybridized to the AH60c DNA probe according to the Grünstein and Hogness procedure (12).

Blot Hybridization Analysis. Fifty micrograms of total poly(A)⁺ RNA from normal liver and AH60c per 1.5 × 10⁶ mm² slot were electrophoresed through a 1.5% agarose/6% (vol/vol) formaldehyde gel and blotted onto nitrocellulose paper (14). The nitrocellulose paper was then baked at 80°C for 2 hr, prehybridized (14), rinsed with 0.3 M NaCl/0.03 M sodium citrate/0.1% Abbreviation: kb, kilobase(s).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
sodium dodecyl sulfate and cut into 6-mm strips. One strip each containing normal liver and AH60c poly(A)⁺ RNA was then hybridized to ³²P-labeled, nick-translated (15) cloned DNA according to the procedure of Thomas (14).

Semiquantitative RNA Dot Blot Assay. A nitrocellulose filter was treated with 3 M NaCl/0.3 M sodium citrate as described by Thomas (14). Serially 1:2-diluted samples of poly(A)⁺ RNA from normal and tumor tissues were spotted in order, dried, baked, and prehybridized. The nitrocellulose filter was then hybridized to ³²P-labeled, nick-translated cloned DNA.

RESULTS

Screening of cDNA Clone Library. We have tried to isolate cDNA clones that exhibit a positive hybridization signal only with tumor mRNA and not with normal mRNA by differential colony hybridization. Approximately 5,000 cDNA clones were prepared from six lines of ascites hepatoma mRNAs. Among this library, about 4,000 clones from four lines of tumors were screened with radiolabeled cDNA made from normal liver and AH60c mRNAs as probes. We selected 160 candidate clones. These clones were then individually streaked on agar plates, and plasmids were isolated by the method described by Holmes and Quigley (16). These plasmids were nick-translated (15) and used for probing nitrocellulose filters on which poly(A)⁺ RNAs from normal liver and AH60c were fixed after size fractionation. Many clones exhibited similar responses to both normal and tumor mRNA on blots. Thirty-one clones, however, showed a strong signal with tumor mRNA but no signal or only a weak one with normal mRNA. A part of such an assay is shown in Fig. 1. These clones were categorized into two characteristic groups: (i) clones complementary to discrete size classes of mRNA and consequently showing a single band on blot hybridization (Fig. 1, lanes A–H), and (ii) clones containing sequences expressed in a wide variety of size classes of mRNA and, as a consequence, showing a smear (Fig. 1, lanes I–L). The first group could further be divided into two subgroups showing hybridization with approximately 1.5-kilobase (kb) (Fig. 1, lanes A–D) and 0.6-kb mRNAs (Fig. 1, lanes E–H). As Table 1 summarizes, these two groups of clones were isolated from the clone libraries made independently from any of the different lines of ascites hepatomas except AH130. Inefficiency of clone isolation from this collection was probably due to general short length of inserts in clones that occurred in this particular library during preparation of double-stranded cDNAs. That there was no selective appearance of a specific group of RNA sequences in a particular line of AH was confirmed later by dot blot assays (Fig. 2). We therefore tentatively considered these clones as tumor specific. We do not, however, know whether or not clones in each group have homologies in their sequences, since only a limited number of clones of a given group have shown a positive response with a ³²P-labeled insert fragment from one of these clones (e.g., 3 out of 9, 5 out of 8, and 4 out of 14 clones of 0.6- to 1.5-kb, and smear group, respectively), probably because of non-full-length of the insert in each clone. We could not decide whether these nonhomologous clones were derived from different species of mRNA or from different parts of the same mRNA.

The smear group seems to contain more than one sequence, since (i) the smear distribution on blots probed with one clone was slightly different from that obtained with another (Fig. 1, lanes J and L), and (ii) the level of expression in various lines of tumor mRNA differs significantly from one clone to another (see below,Fig. 2 D and E).

Expression of the Sequences in Various Tumors. The fact that selected tumor-specific clones fell eventually in these two groups, irrespective of AH lines, strongly indicated that the change in gene expression might be consistent and common to many tumors. To test consistence and the level of expression in various tumor lines, we have carried out semiquantitative RNA dot blot hybridization with poly(A)⁺ RNAs from various sources as in Fig. 2. Poly(A)⁺ RNAs from normal, fetal, and regenerating liver were included as nonmalignant controls. Serially 1:2 diluted samples of poly(A)⁺ RNAs were spotted on nitrocellulose filters in a grid format as shown in Fig. 2A and hybridized with nick-translated clone plasmids representing 1.5-kb, 0.6-kb, or smear group RNA. Results are shown in Fig. 2 B–E. Sequences of all clones selected as in Fig. 1 were expressed in most of the tumor lines, including all seven AH lines, both in ascites form and solid form, Yoshida sarcoma of Donryu rat, Morris hepatoma 5123D of Buffalo rat, and Walker 256 carcinosarcoma of Wistar rat, but much less, if any, in normal tissues. Fig. 2 D and E represents patterns obtained with two clone

![Fig. 1. Gel electrophoretic analysis of normal liver and ascites hepatoma poly(A)⁺ RNAs probed with individual clones. Poly(A)⁺ RNAs were denatured and subjected to electrophoresis in a 1.5% agarose/formaldehyde gel (13). After electrophoresis, the RNA was transferred to nitrocellulose, which was baked, prehybridized, and cut into 6-mm strips representing about 5 μg each of RNA from normal liver (lanes A, C, E, G, I, and K) or AH60c (lanes B, D, F, H, J, and L). The nitrocellulose paper strips were then hybridized to individual cDNA clones separately (specific activity 5 × 10⁶ cpm/μg) for 20 hr. Plasmid DNAs purified by CsCl/ethidium bromide gradient centrifugation were used for this experiment. The autoradiographic exposure time was 48 hr. Cloned DNAs were A and B, pAH26; C and D, pAH34; E and F, pAH29; G and H, pAH35; I and J, pAH43; K and L, pAH53. Positions of ribosomal RNAs are shown on the right.](image-url)

Table 1. Screening of cDNA clone libraries

<table>
<thead>
<tr>
<th>Source of mRNA</th>
<th>Total no. of clones screened</th>
<th>0.6-kb clones isolated</th>
<th>1.5-kb clones isolated</th>
<th>Smear clones isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH60c</td>
<td>2,020</td>
<td>4</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>AH606F</td>
<td>763</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>AH109A</td>
<td>297</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>AH130</td>
<td>849</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3,929</td>
<td>9</td>
<td>8</td>
<td>14</td>
</tr>
</tbody>
</table>

cDNA clone libraries were prepared from mRNAs from six lines of ascites tumor cells. Four independent libraries were screened with differential blot hybridization as described for Fig. 1. Clones containing sequences specific to tumor cells were isolated and divided into three characteristic groups on the basis of the sizes of mRNAs from which clones were derived.
plasmids of the smear group. The sequence of pAH53 was expressed at a high level in almost all tumor mRNAs, with the exception of AH66, which showed significantly less signal than other tumors (Fig. 2E). On the other hand, the expression of the pAH43 sequence greatly fluctuated from one tumor line to another (Fig. 2D). Some AH lines (AH66F and AH44), Yoshida sarcoma, Walker carcinosarcoma, and Morris hepatoma showed practically no signals on RNA dot blots. Clones in the smear group thus appear to be heterogeneous in sequence. It should therefore be noted that, in general, sequences of this group appear preferentially in tumor cell mRNAs, but not all tumor cells necessarily express all of these sequences.

Southern Blot Analysis of Rat Genomic DNA. Unexpected was a smear pattern observed with some of the tumor-specific clones. This result indicated that the sequences in this group of cDNA clones were expressed ubiquitously in a variety of mRNA species. One possible explanation might be the involvement of repetitive sequences dispersed in the genome. We have therefore performed Southern blot analysis of rat genomic DNA. When rat liver and hepatoma DNA was digested with EcoRI or HindIII and then hybridized with nick-translated plasmid DNA of smear clone pAH53, a complex series of bands among considerable smear was observed (Fig. 3). This shows that at least a part of the cDNA insert is repeated in the rat genome. Band patterns obtained from both normal liver and hepatoma DNAs appeared essentially the same for both restriction enzymes, indicating no extensive genomic rearrangements involving the pAH53 sequence, although the possibility of minor change hidden in the background smear could not be eliminated. The presence of bands indicates that a fraction of the sequence arrays in tandemly repeated fashion as well as in dispersed form. Repetition of the sequence was also confirmed by the Benton-Davis screening of rat genomic library packaged in \( \lambda \) Charon 4A phages (obtained from L. Jagodzinsky and J. Bonner) (18).

About 5% of the genomic library showed a positive signal with a cDNA clone (pAH53) of the smear group. The minimal copy number of the sequence was estimated to be approximately 2 \( \times 10^4 \) copies per genome.

**DISCUSSION**

Transformation accompanies a variety of changes in cellular phenotypes. We have made attempts to isolate mRNA sequences expressed in tumor cells at higher abundance than in their normal counterpart for the elucidation of both quantitative and qualitative changes in transcription. Approximately 4,000 cDNA clones were screened by differential colony hybridization with radiolabeled cDNA made from normal and tumor cells. The majority of the clones have revealed the same response to both probes. This indicates strongly that AH lines are indeed derived from hepatic cells and the transcribability of many, if not all, open genes of normal liver cells is retained in transformed cells. Our observations thus appear to support the results of overall similarity in gene expression between normal and tumor cells (1–5). Similar attempts had been made recently with normal rat liver (13), mouse colon tumor (19), and human leukemia cells (20). Considerable differences in mRNA abundance between tumor and normal tissue were observed (19, 20). The apparent discrepancy with our results might be due to the normal counterpart used for different colony hybridization—i.e., for colon tumor, adjacent colon tissue (19), which is much more complex in structure and is composed of a variety
of cell types, and for chronic lymphocytic leukemia cells, placenta tissue (20).

For the isolation of tumor-specific cDNA clones, we have set a criterion of consistent hybridizability with tumor mRNAs but none with normal ones. The selection method employed allowed only the cloning of sequences represented moderately to highly in tumor tissues. It was of interest that isolated clones were categorized into two groups. The sizes of mRNAs complementary to the first group were exclusively either approximately 1.5 kb or 0.6 kb, while those of the second group were multiple, which resulted in a smear pattern on blot hybridization (Fig. 1). The most probable reason for the smear formation is the involvement of common sequences in various kinds of mRNAs. Indeed, the sequence repeats in the rat genome (Fig. 3). Middle-repetitive sequences of DNA are interspersed between unique sequences and are known to be extensively transcribed (21). Davidson and Britten have proposed a model for the regulation of gene expression in which products of interspersed repetitive sequences have a key role through the formation of repetitive RNA–nuclear RNA duplexes (22). Recently, Sutcliffe et al. (23) observed that a specific class of repetitive sequence was transcribed exclusively in rat brain tissue. Since this transcript was not found in other tissues, they hypothesized that this transcript could be an identifier sequence for the transcription of brain-specific mRNAs. The sequences contained in smear clones of this report may be identifiers for tumor-specific mRNA transcription, since they are absent from normal tissues but appear preferentially in many tumor lines tested. If there are such identifiers or transcripts of middle-repetitive sequences responsible for the transcription of tumor-specific mRNAs, it will become possible to explain broad changes in phenotype accompanying neoplastic transformation. Cotranscription of a class of repeated sequences was also reported in Dictyostelium mRNAs obtained at specific stage of development (24).

The appearance of middle-repetitive sequences in poly(A)+ RNA could alternatively be a laboratory artefact due to gene rearrangement caused by long-term serial passage of the tumor in a form of floating cells. However, the form of transplanted cells itself was certainly unrelated to the expression of a specific class of middle-repetitive sequences, since subcutaneously passaged AH cells that grow as a solid mass and serially transplanted solid tumors of other strains such as Walker carcinosarcoma and Morris hepatoma also transcribed the sequences equally well (Fig. 2). The apparent identical genomic arrangement of the pAH53 sequence in both normal and tumor tissues (Fig. 3) seems to support our view that the expression is controlled somewhere around transcription-processing steps that should be coupled to the transformed state of a cell. In any case, it seems very important to examine the expression of these sequences in primary tumor tissues, as has been emphasized recently by Feinberg et al. (25).

We are grateful to Mr. N. Kinoshita and Miss M. Honda for assistance. This work was supported in part by research grants from the Japan Tobacco and Salt Public Corporation, from the Japanese Foundation for Multidisciplinary Treatment of Cancer, and from the Fukuoka Cancer Society.