Construction of a normalized cDNA library by introduction of a semi-solid mRNA – cDNA hybridization system

Yasnory F.Sasaki, Dai Ayusawa and Michio Oishi*

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

Received December 23, 1993; Revised and Accepted February 16, 1994

ABSTRACT

We report a novel procedure to construct a normalized (equalized) cDNA library. By introduction of the highly efficient self-hybridization system between a whole mRNA population and their corresponding cDNA immobilized on latex beads, which involves relatively simple manipulations, we were able to generate an mRNA population in which the copy number of abundant species was reduced while that of rare species was enriched. In a typical experiment, after several cycles of self-hybridization on the beads, the ratio of the most to the least abundant marker mRNA species dropped by a factor of 300 (from 10,000 to 30) while the complexity and length of mRNAs in the population remained unchanged. The procedure should provide a potent tool for the expression cloning of cDNA and also facilitate the construction of whole cDNA catalogues from specific tissues (or cell types) from higher organisms.

INTRODUCTION

In most tissues and cells, the factor of variation in the copy number of the most and the least abundant mRNA species is estimated to be more than 100,000¹. It therefore follows that the majority of cDNA clones in a given unmodified library represents those of very abundant and moderately abundant classes of mRNA species. For the efficient cloning of cDNA belonging to a rare mRNA class as well as for the construction of a whole human cDNA catalogue, one of the major human genome projects, it is quite important to establish a library in which the frequency of the occurrence of every cDNA clone is equal (an equalized or normalized library). Furthermore, if the cDNA library thus constructed includes full-length cDNA clones, the usefulness of such a library would be multiplied, for the library can be directly used for the expression cloning of rare cDNA clones.

Only a limited number of attempts, however, have been made to construct a normalized cDNA library. Among them, Ko² and Patanjali *et al*³ independently reported that repeated cycles of self-hybridization of fragmented cDNA molecules and removal of reassociated DNA resulted in normalization of the clones in

the cDNA library. The rationale behind these approaches is that DNA reassociation follows a second-order kinetics as such that abundant species reassociate faster than rare species, leaving a pool of unhybridized single-stranded DNA rich in rare species. Under these conditions, however, parity in copy number of cDNA representing the most and least abundant mRNA species can be obtained only under theoretically optimal conditions and was not reached under those contrived in the laboratory. More importantly, the cDNA libraries were made up only with short cDNA inserts since artificially fragmented cDNA molecules rather than the original cDNA molecules were used.

In this article, we present a theoretically as well as technically different approach to normalize an mRNA population by introducing a very efficient semi-solid system to achieve self-hybridization even under low Rot conditions, and at the same time to effectively remove abundant class of mRNA species which follows a pseudo first-order kinetics. We also took it into consideration that the normalized cDNA library is to contain full-length cDNA clones. In addition, our procedure was designed also for the concentration of mRNA species unique to specific tissues or cell types. Here we present the underlying principles and outline of the novel procedure for the construction of normalized cDNA libraries.

MATERIALS AND METHODS

Enzymes and reagents

Enzymes were purchased from Takara Shuzo unless otherwise specified. Rabbit β -globin (β -glo) and neomycin resistant (*neo*^r) poly(A)⁺ RNA were purchased from Amersham and Boehringer Mannheim, respectively. Plasmids pSP64 ϕ X0.6 and pSP64 ϕ X0.9 were kindly supplied by Dr. M. S. H. Ko (Wayne State Univ.).

Cell lines

Chinese hamster ovary (CHO)^{4,5,6} and human diploid fibroblast cells (TIG-7)⁷ were cultured in a CO₂ incubator at 37°C in plastic dishes in ES medium (Nissui Seiyaku) supplemented with 5% (CHO) and 10% (TIG-7) fetal calf serum (FCS, Cell Culture Laboratories), respectively.

*To whom correspondence should be addressed.

Preparation of poly(A)⁺ RNA

Poly(A)⁺ RNA from CHO and TIG-7 cells were prepared by the guanidium isothiocyanate method⁸, followed by oligo (dT) cellulose (type 7, Pharmacia) chromatography⁹. pSP64AφX0.6 and pSP64AφX0.9 which carry 603 bp and 872 bp φX174 *Hae*III fragments, respectively, were linearized by *Eco*RI and transcribed with SP6 RNA polymerase to yield poly(A)⁺ φX174 0.6 kb and 0.9 kb RNA. The transcripts were then subjected to oligo (dT) cellulose chromatography, treated with phenol/chloroform and precipitated with ethanol.

Conversion of mRNA to cDNA on latex beads

Twenty μg of poly(A)⁺ RNA was mixed with 2.5 mg oligo (dT) 30-conjugated latex beads (Oligotex-dT30, Nippon Roche, supplied through Japan Synthetic Rubber) in 250 μl of a buffer (50 mM Tris-HCl, pH 8.3 at 42°C, 10 mM MgCl₂ and 100 mM KCl). The mixture was incubated for 20 min at 37°C, centrifuged at 15,000×g for 10 min at room temperature and the precipitated mRNA/Oligotex complexes were resuspended in 250 μl of a buffer (50 mM Tris-HCl, pH 8.3 at 42°C, 6 mM MgCl₂ and 40 mM KCl). Reverse transcription was then performed on the beads in a reaction mixture (500 μl) containing 50 mM Tris-HCl, pH 8.3 at 42°C 6mM MgCl₂, 40 mM KCl, 1 mM each of dATP, dCTP, dGTP and TTP, 750 units AMV reverse transcriptase (Seikagaku Kogyo), 4 mM DTT and 300 units RNase inhibitor (Takara Shuzo) by incubating the mixture for 90 min at 42°C, as described by Kuribayashi *et al.*^{10,11} and Hara *et al.*¹². The beads were washed twice with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), heated for 3 min at 95°C to remove RNA, centrifuged, resuspended in TE 10 mM Tris-HCl, pH7.5, 1 mM EDTA and stored at 4°C until use. To monitor cDNA synthesis on the beads, a small portion of the mixture was incubated in parallel with ³²P-labeled dCTP (111 TBq/mmol; ICN) and bead-associated radioactivities were counted.

mRNA-cDNA self-hybridization on the beads

mRNA-cDNA self hybridization was performed as reported^{11,12} with slight modifications. The beads (cDNA-Oligotex complex, 2.5 mg) were suspended in 90 μl of TE containing 100 μg of oligodeoxyadenylates (25–30 mer) (Pharmacia) and heated for 5 min at 70°C. After adding 10 μl of 5 M NaCl, the suspension was incubated for 10 min at 37°C to mask oligo (dT) residues. After removing the excess oligodeoxyadenylates by centrifugation, the beads were incubated with poly(A)⁺ RNA (2 μg) in 200 μl of a hybridization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl) for 15 min at 55°C with occasional shaking. The beads were then removed by centrifugation (15,000×g, 10 min) and the supernatant fraction was likewise hybridized with the same amount of new or regenerated cDNA-bead complexes when indicated (see below). After treating the supernatant with phenol/chloroform, RNA was precipitated with ethanol, dried and dissolved in H₂O.

To regenerate cDNA-Oligotex complex, the beads were resuspended in 200 μl of TE, heated for 5 min at 70°C, chilled on ice and washed twice with TE and resuspended in TE.

RT (reverse transcription)-PCR

Reverse transcription was achieved with ca. 1 ng of poly(A)⁺ RNA and 500 ng of oligo (dT) cellulose by incubating for 60

min at 37°C in a 20 μl reaction mixture containing 200 units of Superscript (BRL). The mixture was diluted with TE, boiled for 2 min and subjected to PCR^{13,14}. The PCR mixture (50 μl) contained cDNA (see text and also Figure legends), 0.2 mM each of dATP, dGTP, dCTP and TTP, 1 μM 5' and 3' primers, 1 unit Taq DNA polymerase (Promega), 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin and 0.1% Triton X-100. Each PCR cycle consisted of 94°C (1 min), 57°C (2 min) and 72°C (3 min). The primer sets used were 5'-CATGGTGGAAAATGGCCGCT-3' and 5'-GAAGAACTCGTCAAGAAGGC-3' for *neo*^r cDNA marker¹⁵, and 5'-ATGTGTGCATCTGTCCAGTG-3' and 5'-TCAGGATCCACGT GCAGCTT-3' for *β-glo* cDNA marker¹⁶.

Construction of cDNA libraries

Poly(A)⁺ RNA mixtures were annealed to a primer-adaptor (5'-GAAGAAGAACTCGAGGGTACCTTTTTTTTTTTTTTTT-3'), which contained 15 dT residues and two restriction endonuclease sites (*Xho*I, *Kpn*I). The first-strand cDNA was synthesized with MMLV reverse transcriptase (Superscript), and the second-strand cDNA with 1.5 units *E. coli* RNase H, 37.5 units *E. coli* DNA polymerase I, 10 units *E. coli* DNA ligase, 50 mM Tris-HCl (pH 6.9), 5mM MgCl₂, 100 mM KCl, 5 mM DTT, 10 mM (NH₄)₂SO₄ and 0.1 mM β-NAD⁺¹⁷. The double-stranded cDNA was blunted with T4 DNA polymerase, ligated to *Eco*RI-*Not*I-*Bam*HI adapters (Takara Shuzo) and phosphorylated. The DNA was purified through a Sephacryl S-400 column (Pharmacia) and cloned into the λgt10 vector (Stratagene)¹⁸.

Screening of phage clones

Phage plaques were transferred onto nylon membranes (Hybond-N⁺; Amersham) as suggested by the manufacturer and hybridized with probes labeled with α-³²P dCTP (111TBq/mmol; ICN) using a randomly primed DNA labeling kit (Boehringer Mannheim) in a mixture containing 50% (v/v) formamide, 5×SSC, 5×Denhardt's reagent, 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 0.1 mg/ml sonicated herring sperm DNA for 16 hrs (or 72 hrs) at 42°C. The membranes were washed twice with 0.1×SSC/0.1% SDS for 30 min at 65°C and exposed to autoradiographic films (XAR-5, Kodak) for 16 hrs at -80°C with an intensifying screen.

Positive plaques were picked up and subjected to PCR using a primer set for λgt10:(5'-GCTGGGTAGTCCCCACCTTT-3' and 5'-CTTATGAGTATTTCTTCCAGGGTA-3'). The inserts were excised with *Xho*I and *Not*I, cloned into pBluescript (Stratagene) and sequenced by the dideoxy chain termination method¹⁹ using the Sequenase ver. 2.0 Sequencing kit (United States Biochemical).

RESULTS

Theoretical basis

In the hybridization process between two complementary DNA (or RNA) strands, the concentration (C) of unhybridized DNA or RNA at a given time (t) is expressed as $C = C_0 / (1 + kCot)$, in which C_0 is the initial concentration of DNA (RNA) species and k is a reaction constant^{20,21,22,23}. This was the theoretical basis for the construction of the normalized cDNA libraries reported previously^{2,3}. According to this equation, however, although the concentration of the remaining unhybridized DNA (RNA) representing the more abundant species decreases at a greater rate than that of less abundant species, the concentrations

of unhybridized DNA (RNA) species become equal only at the time of the completion of the hybridization. On the other hand, when the initial concentration of one DNA (RNA) strand (D_0) is greater than that of the complementary strand (R_0), the relative concentration of the unhybridized complementary strand (R) at t is given by the equation $R = R_0 e^{-k D_0 t}$ [22,23]. In this case, the rate of decrease of the unhybridized DNA (RNA) species (R) greatly depends on the initial concentration of the complementary strands of DNA (RNA) (D_0), indicating that abundant DNA(RNA) species reassociate much faster in this system than

Figure 1. Kinetics of hybridization between mRNA and corresponding cDNA immobilized on latex-beads. Two micrograms of ^3H -labeled mRNA from CHO cells was incubated with corresponding cDNA ($\sim 2 \mu\text{g}$) immobilized on the latex beads in a hybridization buffer (200 μl) for 15 min at 55°C . At intervals, the samples were withdrawn, beads-associated radioactivities were counted in a scintillation counter and expressed as the percentage of the input mRNA. For details, see Methodology.

Figure 2. Hybridization of mRNA to bead-associated cDNA. ^3H -labeled poly(A) $^+$ RNA (2 μg) from CHO cells and standard mRNA (see below) were incubated with corresponding cDNA (synthesized from 20 μg of the mRNA) associated with latex beads for 15 min at 55°C in a hybridization buffer (200 μl). The beads were then removed by centrifugation and the supernatant was again hybridized using the same, but regenerated bead-associated cDNA. The process was repeated (total 4 times). Radioactivities in the supernatant were normalized as the percentage of mRNA remaining in the unhybridized fraction. Samples (n1, n2 and n3) included mRNA plus different amount of *neo^r* RNA, 10 ng (n1), 100 pg (n2) and 1 pg (n3) per μg total RNA, and a constant amount of β -*glo* RNA (1 ng per μg RNA) to all the samples.

in the previously reported systems. To adopt this, however, we had to find a condition in which two complementary DNA (RNA) strands are efficiently hybridized at unequal molar ratio. A recently developed solid-phase matrix on which cDNA can be immobilized was thus introduced and this allowed us to construct a normalized cDNA library with unique properties as described below.

mRNA–cDNA self-hybridization on latex beads

We first examined the kinetics of hybridization between cellular mRNA species and cDNA immobilized on the latex beads. As shown in Figure 1, the hybridization reaction was very rapid and reached a plateau in less than 10 min at practical cDNA (at excess) and RNA concentrations. In subsequent model experiments, we found, as expected, that abundant poly(A) $^+$ RNA species in the RNA mixture were preferentially removed by self-hybridization with their corresponding cDNA immobilized on the latex beads. For this, ^3H -labeled poly (A) $^+$ RNA was prepared from cultured CHO cells and divided into three equal portions. To each, various amounts of *neo^r* RNA (at the concentration of 10 ng, 100 pg and 1 pg per μg RNA) as well as a constant quantity of β -*glo* RNA (1 ng/ μg RNA) were added as standard RNA markers. A portion of each sample was then converted to cDNA on the latex beads as described in Methodology section. The rest of each RNA samples (2 μg each) were incubated with the corresponding bead-associated cDNA (ca.2 μg cDNA) for 15 min at 55°C . Since the average molecular size of the cDNAs was approximately 500 bp, the equal amount of the cDNA should be 4-fold molar excess to that of the input RNA sample (ca.2.0 kb on average). Under these conditions substantial amounts (70 to 80%) of the RNA as measured by their radioactivities were associated with the beads after the first cycle of hybridization (Figure 2). The unhybridized fraction was subjected to the second-cycle of self-hybridization with the same amount of regenerated cDNA library used in the first cycle. After 4 cycles of hybridization-subtraction, 96 to 98 % of the input poly(A) $^+$ RNA was reproducibly removed, leaving 2 to 4 % of the RNA in the unhybridized supernatant fraction.

We assayed relative concentrations of the exogenous standard RNA markers (*neo^r* and β -*glo* RNA) in the RNA samples after each cycle of self-hybridization by RT-PCR (the sensitivity of Northern blot hybridization was insufficient to detect the RNA markers at low concentrations). Fig 3A shows the change in the relative concentration of the *neo^r* RNA marker in the supernatants after 0, 1 and 4 cycles of self-hybridization. The concentration of the *neo^r* RNA was markedly decreased in n1 sample (10 ng/ μg RNA), not significantly changed in n2 (100 pg/ μg RNA), and greatly increased in n3 (1 pg/ μg RNA) after 4 cycles of self-hybridization. Fig 3B shows that the β -*glo* RNA, which was present at an equal and relatively high concentration (10^{-3} ; 1 ng/ μg RNA) in all samples, decreased similarly in n1, n2 and n3 samples as did the *neo^r* RNA in n1 sample. Thus, under the condition described here, self-hybridization between an mRNA population and an excess its cDNA associated with the latex beads efficiently removed the most and intermediate abundant class of mRNA species which existed more than 10^{-4} (w/w) and concomitantly increased the rare class of mRNA.

Construction of the normalized human cDNA library

Poly (A) $^+$ RNA sample was prepared from human diploid fibroblasts (TIG-7) and added with the exogenous RNA markers at the concentrations (per μg RNA) as indicated; β -*glo* RNA,

Figure 3. Analysis of *neo^r* and *β-globin* RNA before and after hybridization. Equal amounts of RNA as measured by their radioactivities in the supernatant fractions after 0 (control), 1 and 4 self-hybridization cycles of the poly(A)⁺ RNA from CHO cells (see the legend to Figure 2) were converted to cDNA. Samples, n1, n2 and n3 originally contained different amounts (10 ng, 100 pg and 1 pg per μ g total RNA, respectively) of *neo^r* RNA plus a constant amount *β-globin* RNA concentration (1 ng/ μ g RNA) as standard RNA markers. The cDNA was subjected to PCR using either *neo^r* or *β-globin* specific primers in 50 μ l of the PCR reaction mixture. Because of wide variations in the mRNA concentrations in each sample, different template concentrations as well as PCR cycles were used. For *neo^r* RNA, n1, one thousandth of the cDNA was used as the PCR template (25 PCR cycles); n2 and n3, one tenth of the cDNA was used (30 PCR cycles). For *β-globin* RNA, n1 to n3, one thousandth of the cDNA was used (25 PCR cycles). In both reverse transcription and PCR, the template concentrations were in the range where the amounts of their products were proportional to the template concentrations. (A), *neo^r* RNA; (B), *β-globin* RNA. The size of the amplified DNA fragments are also indicated. Lane M, molecular size markers (1 kb DNA ladder; BRL).

10 ng; ϕ X174 0.6 kb and 0.9 kb RNA, each 100 pg; and *neo^r* RNA, 1 pg. The RNA sample (2.0 μ g) was self-hybridized with the equivalent bead-associated cDNA ($\sim$ 2.0 μ g) which corresponded to an approximately 4-fold molar excess to the RNA. Changes in the relative concentrations of the *β-globin* RNA and *neo^r* RNA markers were examined by RT-PCR. As shown in Figure 4, after 4 cycles of self-hybridization, the concentration of the *β-globin* marker decreased by a factor of over 100, whereas that of the *neo^r* marker increased by a factor of 17.

We then constructed phage libraries from the control (0 cycle) and the self-hybridized (4 cycles) mRNA samples and determined the frequency of the occurrence of the exogenous and several endogenous RNA markers by plaque hybridization analysis. When the *β-globin* cDNA was used as a probe, the number of positive plaques dramatically decreased after 4 cycles of self-hybridization (Figure 5A and B), confirming the results obtained by RT-PCR. Similarly, a sharp decrease in the number of positive plaques were observed when the total cDNA inserts from the control (0 cycle) library was used as the probe (Figure 5C and D). This indicated that the abundant endogenous mRNA species present in human fibroblasts were removed as efficiently as the exogenous abundant *β-globin* RNA marker after self-hybridization. On the other hand, when cDNA inserts from the self-hybridized

Figure 4. Alteration of the *β-globin* and *neo^r* RNA levels before and after hybridization. Poly (A)⁺ RNA was prepared from cultured human fibroblasts (TIG-7) and the following standard RNA markers were added to the RNA sample at the concentrations (per μ g RNA) indicated. *β-globin* RNA, 10 ng; ϕ X174 0.6 kb RNA, 100 pg; ϕ X174 0.9 kb RNA, 100 pg and *neo^r* RNA, 1 pg. The RNA sample was hybridized with corresponding bead-associated cDNA ($\sim$ 2.0 μ g) which had been prepared from the RNA sample. After 0, 1 and 4 cycles of hybridization, the supernatant was converted to cDNA in the presence of [α -³²P] dCTP. After normalization, equal amounts of the cDNA were then subjected to PCR in the presence of [α -³²P] dCTP (25 cycles for *β-globin* RNA and 35 cycles for *neo^r* RNA) using *β-globin* or *neo^r* specific primers in a 50 μ l reaction mixture. The PCR products were then electrophoresed, autoradiographed and visualized using an image analyzer (BAS2000; Fuji) (top panel). The relative radioactivities in the hybridized spots are shown (at the bottom panel).

(4 cycles) library were used as a probe, no clear plaque was observed in either of the libraries (data not shown). Phage plaques were also screened with DNA probes for other exogenous and several endogenous RNA markers (Table 1). Among the exogenous RNA markers, the frequency of the most abundant marker *β-globin* RNA decreased from 111/10,500 (1.067 %) to 5/55,000 (0.009 %) after 4 cycles of self-hybridization. The frequency of the least abundant marker *neo^r* RNA originally present at 10^{-6} (w/w) increased from an undetectable level (less than one per 250,000) to 0.0008 % (2/250,000). The frequency of the moderately abundant markers, ϕ X174 HaeIII 0.6 kb and 0.9 kb fragments, increased slightly by 3–6 fold. The frequency of endogenous *β-actin* cDNA²⁴ belonging to abundant class decreased by a factor of 27 from 54/10,000 (0.54%) to 2/10,000 (0.02%). Several rare house-keeping cDNA clones such as for thymidylate synthase cDNA, hypoxanthine phosphoribosyl transferase, thymidylate and thymidine kinase were also enriched in the normalized library (not shown). We examined whether interleukin 2 (IL-2)²⁵ and 4 (IL-4)²⁶ cDNA clones, thought to be present at extremely low levels in the cells, could be found in the self-hybridized library. While no IL-2 clones were found in either of the control or self-hybridized library, three IL-4 clones were detected in the self-hybridized library.

Molecular size of the cloned cDNA

In order to examine whether our procedure causes any degradation of mRNA, we compared the size distribution of cDNA inserts of the original library with that of the four cycled normalized. As shown in Figure 6, the electrophoretic patterns

Figure 5. Plaque hybridization of phage cDNA libraries. Phage lambda libraries were constructed from cDNA samples prepared by reverse transcription from mRNA (products of 0 and 4 cycles of hybridization, see the legend to Figure 4). Plates (each with 1×10^4 plaques) were plaque-hybridized using ^{32}P -labeled β -glo or total cDNA as probes. The total cDNA probe was prepared by labeling cDNA inserts (by random priming) from the control (0 cycle) sample. (A), control (0 cycle) sample hybridized with β -glo probe; (B), 4 cycles hybridized with β -glo probe; (C), control (0 cycle) sample hybridized with the total cDNA probe and (D), 4 cycles sample hybridized with total cDNA probe.

Change of the proportion of cDNA clones before and after self-hybridization					
Probe	Input *	Before (%)	After (%)	After/Before	
rabbit β -globin	1	111/10,500 (1.067)	5/55,000 (0.009)	0.0086	
Φ X174Hae III 0.6 kb	0.01	2/30,000 (0.0067)	8/35,000 (0.023)	3.43	
Φ X174Hae III 0.9 kb	0.01	1/30,000 (0.0033)	6/30,000 (0.02)	6	
neo ^r	0.0001	0/250,000 (<0.0004)	2/250,000 ** (0.0008)	>2	
β -actin		54/10,000 (0.54)	2/10,000 (0.02)	0.037	
IL-4		0/320,000 (<0.0003)	3/320,000 ** (0.0009)	>3	
IL-2		0/320,000 (<0.0003)	0/320,000 (<0.0003)	—	

* Percent of total RNA (w/w)

**The positive clones were confirmed by sequencing approximately 300bp of the inserts.

of the inserts from the two libraries, ranging from 0.5 to 3.0 kb, were indistinguishable. No signs of degradation of specific mRNAs after having subjected to the procedure were also detected by Northern blot hybridization analysis (data not shown). In fact, the size of the inserts of several β -actin clones (2.1 kb) and three IL-4 clones (0.6 kb) isolated from the self-hybridized library was the same as those reported for the full length cDNAs with intact sequences of the 5'-untranslated region (data not shown).

DISCUSSION

In this paper, we have described a procedure to construct a normalized cDNA library. The procedure is, theoretically and technically, different from those reported previously ^{2,3}, which employed repeated dissociation-reassociation of double-stranded short cDNA fragments in solution. In contrast, we took an advantage of using cDNA immobilized on latex beads, which provided highly efficient hybridization between mRNA and

Figure 6. Size of the cDNA inserts before and after hybridization. DNA from lambda phage libraries constructed from mRNA after 0 and 4 cycles of hybridization (see the legend to Figure 4) was treated with *Eco*RI and *Xho*I, electrophoresed through 1% agarose gel and stained with ethidium bromide. Lane 1, control sample (0 cycle); lane 2, hybridized sample (4 cycles). The DNA bands corresponding to the phage vector are indicated.

corresponding cDNA with different molar ratios even under low Rot conditions and made it quite easy to enrich rare mRNA species. The procedure is much simpler and less time-consuming than those reported previously. More importantly, perhaps because of the simple manipulations, the cDNA species in the library maintained the original molecular structures including full length cDNA molecules, which is essential in isolating cDNA clones in transfected cells by functional expression assays. Although, theoretically, abundant mRNA species could be completely removed from the library after repeated self-hybridization cycles, the degree of normalization can be controlled by simply changing the number of hybridization cycles and/or the molar ratio of cDNA on the beads to mRNA in solution.

Our procedure, like others, which depends upon self-hybridization seems to embrace following inherent problems. First, since a certain fraction (ca.10%) of poly(A)⁺ RNA samples contains *Alu* sequence in non-coding regions, hybridization between authentic complementary strands may be interfered by such DNA molecules. This problem, however, can be easily solved by adding excess *Alu* sequences. Second, when a homologous sequence happens to be commonly present in rare as well as abundant mRNA species alike, cross-hybridization between them could eliminate the rare RNA species. One way to circumvent this problem would be to use an immobilized cDNA library consisting of mostly 3'-untranslated region. In the experiments described above, whereas self-hybridization between mRNA and cDNA was employed, the efficacy of employing cDNA-cDNA hybridization, using cDNA instead of mRNA, remains an open question. Preliminary experiments, however, indicated that the extent of the enrichment of rare cDNA by cDNA-cDNA self-hybridization was not as great as that for mRNA-cDNA self-hybridization for unknown reasons.

Several means may be considered to increase the efficiency of enrichment of rare mRNA species to even higher levels. For example, through our experiments described here, we maintained the initial ratio of cDNA to mRNA during self-hybridization to approximately 4 to 1. Although the ratio increased progressively after each cycle of self-hybridization as a result of removal of hybridized poly(A)⁺ RNA, use of even a larger excess of bead-associated cDNA should eliminate abundant mRNA species more

efficiently, thus resulting in even greater enrichment of rare mRNA species. Alternatively, the degree of enrichment of rare mRNA species may also be improved if greatly accelerated hybridization conditions are found. Use of hybridization enhancers such as polyethylene glycol²⁷ or cetyltrimethyl ammonium bromide²⁸ may be considered for future research.

Needless to say, our procedure would be also of great use in constructing a whole human cDNA catalogue. Although attempts to make human cDNA catalogues from conventional unmodified cDNA libraries are already in progress in several laboratories²⁹ we will soon encounter a situation where rare classes of mRNA species are more and more difficult to be cloned. In this respect, our procedure would be particularly useful when we reach that stage in near future. Furthermore, even when the whole cDNA catalogue has been made, it will be necessary to assign an individual cDNA clone to specific tissues where it is expressed. Applications of our procedure to the hybridization of cDNA and RNA from two different biological sources should facilitate to construct cDNA library which primarily represents specific mRNA species present only in one of the samples.

ACKNOWLEDGEMENTS

We thank Drs. K. Oda and E. Hara (Science Univ. of Tokyo), and Drs. S. Tamatsukuri, C. Miyamoto and Y. Furuichi (Nippon Loche, Co.) for valuable information regarding the use of Oligotex. We also thank Dr. M.S.H.Ko for providing us standard markers and Ms. T. Kobayashi for her help in preparing the manuscript. This work was supported by a grant-in-aids for the human genome research from the Ministry of Education, Culture, and Science of Japan.

REFERENCES

1. Bishop, J. O., Morton, J. G., Rosbach, M. and Richardson, M. (1974) *Nature* **250**, 199–204.
2. Ko, M. S. H. (1990) *Nucleic Acids Res.* **18**, 5705–5711.
3. Patanjali, S. R., Palimoo, S. and Weissman, S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1943–1947.
4. Puck, T. T., Cieciura, S. J. and Robinson, A. (1958) *J. Exp. Med.* **108**, 945–955.
5. Bradley, W. E. C. and Letovanec, D. (1982) *Somat. Cell Genet.* **8**, 51–56.
6. Nalbantoglu, J., Hartley, D., Phear, G., Tear, G. and Meuth, M. (1986) *EMBO J.* **5**, 1199–1204.
7. Yamamoto, K., Kaji, K., Kondo, H., Matsuo, M., Shibata, Y., Tasaki, Y., Utakoji, T. and Ooka, H. (1991) *Exp. Gerontol.* **26**, 525–540.
8. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
9. Aviv, H. and Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
10. Kuribayashi, K., Hikata, M., Hiraoka, O., Miyamoto, C. and Furuichi, Y. (1988) *Nucleic Acids Res. Symposium Series* **19**, 61–64.
11. Kuribayashi-Ohta, K., Tamatsukuri, S., Hikata, M., Miyamoto, C. and Furuichi, Y. (1993) *Biochim. Biophys. Acta* **156**, 204–212.
12. Hara, E., Kato, T., Nakada, S., Sekiya, S. and Oda, K. (1991) *Nucleic Acids Res.* **19**, 7097–7104.
13. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) *Science* **239**, 487–491.
14. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual/ Second Edition*. Cold Spring Harbor Laboratory Press, New York.
15. Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B. and Schaller, H. (1982) *Gene* **19**, 327–336.
16. Efstratiadis, A., Kafatos, F. C. and Maniatis, T. (1977) *Cell* **10**, 571–585.
17. Gubler, U. and Hoffman, B. J. (1983) *Gene* **25**, 263–269.
18. Huynh, T. V., Young, R. A. and Davis, R. W. (1985) In Glover, D. M. (ed.) *DNA cloning: A practical approach*. IRL Press, Oxford, Vol. I, pp 49–78.
19. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
20. Britten, R. J. and Kohne, D. E. (1968) *Science* **161**, 529–540.
21. Britten, R. J., Graham, D. E. and Neufeld, B. R. (1974) *Methods Enzymol.* **29**, 363–418.
22. Britten, R. J. and Davidson, E. H. (1985) In Hames, B. D. and Higgins, S. J. (ed.), *Nucleic Acid Hybridisation -A Practical Approach*. IRL Press, Oxford, pp. 3–15.
23. Young, R. A. and Anderson, M. L. M. (1985) In Hames, B. D. and Higgins, S. J. (ed.), *Nucleic Acid Hybridisation—A Practical Approach*. IRL Press, Oxford, pp. 47–71.
24. Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6133–6137.
25. Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J. (1983) *Nature* **302**, 305–310.
26. Yokota, T., Otsuka, T., Mosmann, T., Banchereau, J., DeFrance, T., Blanchard, D., De Vries, J., Lee, F. and Arai, K. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5894–5898.
27. Yokota, H. and Oishi, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6398–6402.
28. Pontius, B. W. and Berg, P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8237–8241.
29. Editorial (1992) *Nature Genet.* **2**, 167–168.