Nucleic Acids Research, Vol. 19, No. 25 7097–7104

Subtractive cDNA cloning using oligo(dT)$_{30}$-latex and PCR: isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells

Eiji Hara[1], Tomohiro Kato, Susumu Nakada[2], Souei Sekiya[3] and Kinichiro Oda[2]*
Institute of Medical Science, St Marianna University School of Medicine, Kawasaki 216, Departments of [1]Applied Biological Science and [2]Biological Science and Technology, Science University of Tokyo, Chiba 278 and [3]Department of Obstetrics and Gynaecology, Chiba University School of Medicine, Chiba 280, Japan

ABSTRACT

The human embryonal carcinoma cell line NEC14 can be induced to differentiate by the addition of 10^{-2}M N,N'-hexamethylene-bis-acetamide (HMBA). A subtractive cDNA library specific to undifferentiated NEC14 cells was constructed using oligo(dT)$_{30}$-Latex and polymerase chain reaction (PCR). The method was designed to improve the efficiency of subtraction and the enrichment of cDNA clones corresponding to low abundance mRNAs. The single strand of cDNA was made from mRNA prepared from the HMBA-treated NEC14 cells using an oligo(dT)$_{30}$ primer covalently linked to Latex particles. After removal of the mRNA template by heat-denaturation and centrifugation, the subtractive hybridization was carried out between the cDNA-oligo(dT)$_{30}$-Latex and mRNA from untreated NEC14 cells. Unhybridized mRNA collected by centrifugation was hybridized repeatedly to the cDNA-oligo(dT)$_{30}$-Latex and subtractive mRNA was converted to cDNA. The subtractive cDNA was then amplified by PCR and cloned into pBluescript II KS$^-$. The cDNA library thus constructed consisted of approximately 10,000 independent clones with cDNA inserts of 1.7 Kb on average. Differential hybridization of these transformants indicated that approximately 3% of them contained cDNA inserts specific to the undifferentiated EC cells, some of which were derived from low abundance mRNAs.

INTRODUCTION

The processes of cellular growth and differentiation are highly regulated by a program of differential gene expression. Most of these genes are expressed transiently in response to specific signals and the cells at any stage contain distinctive species of gene products. Isolation of such differentially expressed genes of unknown sequences has been achieved by differential screening of genomic or cDNA libraries with cDNA probes prepared from the cells in two different stages (1). This procedure is, however, very laborious and a huge number of recombinant phages or plasmids must be screened (2,3,4). Alternatively, a subtractive cDNA library can be constructed with stage-specific mRNA which has been enriched by removing mRNA common to the cells in two different stages. Elimination of the common mRNA has been performed by cDNA-mRNA subtractive hybridization followed by a hydroxyapatite chromatography which specifically adsorbs cDNA-mRNA hybrids and free mRNA. The unadsorbed cDNA was then used for construction of a subtractive cDNA library (5,6,7). This procedure, however has considerable disadvantages. It requires large amount of mRNA, and the unhybridized mRNA recovered after chromatography is diluted. In addition, the chromatographic separation procedure requires high temperature, e.g. 60°C which presumably increases the frequency of mRNA degradation.

In the present paper we constructed a subtractive cDNA library specific to undifferentiated human embryonal carcinoma (EC) cells using oligo(dT)$_{30}$-Latex particles and the polymerase chain reaction (PCR) (8). The fine latex particles with a large surface area form a milky suspension and can be easily recovered by centrifugation. The oligo(dT)$_{30}$-Latex was constructed by covalent linkage of oligo(dC)$_{10}$(dT)$_{30}$ at its 5'- proximal region to the carboxyl residues on the surface of the Latex particles (9). About 0.2 nmol of oligo(dT)$_{30}$ molecules were bound to one mg of Latex particles. The poly (A)$^+$ mRNA can be annealed efficiently (~95%) to oligo(dT)$_{30}$-Latex in a short reaction period (10 min) and the cDNA synthesis was carried out with AMV reverse transcriptase using the annealed mRNA as the template and the covalently-linked to oligo(dT)$_{30}$ as the primer (9). The subtractive hybridization was carried out in an eppendorf tube between the cDNA linked to oligo(dT)$_{30}$-Latex and mRNA prepared from another cell type and the unhybridized mRNA was separated by a brief centrifugation at low temperature. The subtractive mRNA was efficiently enriched by successive hybridization of unhybridized mRNA to the cDNA-oligo(dT)$_{30}$-Latex in a relatively short time period and subsequently amplified by PCR after conversion to cDNA. The deviation of amplified cDNA sequences from the original mRNA

* To whom correspondence should be addressed

population was minor at least up to 3 kb length and the undifferentiated cell specific cDNA clones corresponding to low abundance mRNAs were successfully isolated.

MATERIALS AND METHODS

Cell culture

The human embryonal carcinoma (EC) cell line NEC14, derived from a testicular germ cell tumor (Fig.1A) was cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (10). Differentiation of NEC14 cells was induced by the addition of 10^{-2}M N,N'- hexamethylene-bis acetamide (HMBA) (11,12).

Construction of a subtractive cDNA library

The procedure for construction of a subtractive cDNA library is schematically illustrated in Fig.2. NEC14 cells treated with HMBA for 16 h are represented as cell type A and undifferentiated NEC14 cells as cell type B.

(a) cDNA synthesis using oligo(dT)30-Latex. Total cellular RNA was prepared from sub-confluent cultures of undifferentiated (untreated) and HMBA-treated (16 h) NEC14 cells using the method of guanidium isothiocyanate-cesium trifruoroacetate precipitation (13). Poly $(A)^+$ RNA was isolated by affinity chromatography on a oligo(dT)-cellulose column (Collaborative Research Inc. Type 3) (14). Poly (A)+RNA(20 μg) from NEC14 cells treated with 10^{-2}M HMBA (cell type A) was dissolved in 50 μl of H_2O and mixed with 50 μl of 5% (w/v) oligo$(dT)_{30}$-Latex suspension (oligotex-dT30; Nippon Roche Co., Ltd., Japan Synthetic Rubber Co.,Ltd.) (9) in an eppendorf tube, and heated at 70°C for 5 min followed by rapid cooling in ice-water. After addition of an equal volume of 2× TMK buffer (100 mM Tris-HCl(pH 8.3)), 200 mM KCl, 20 mM $MgCl_2$), the mixture was incubated at 37°C for 20 min and centrifuged at 15000 rpm for 10 min at room temperature. The precipitate (mRNA-oligotex-dT30 complex) was dissolved in 400 μl of RT buffer (50 mM Tris-HCl (pH8.3)), 30 mM KCl,8 mM $MgCl_2$, 2mM each of dNTPs) containing 300 U of RNase inhibitor and 1000U of AMV reverse transcriptase (Seikagaku-kogiyo Co.,Ltd. No 120248). An excess amount of reverse transcriptase was added to cover the surface of the Latex particles instead of adding bovine serum albumin (BSA). The reaction was carried out at 37°C for 1.5 h. The reaction mixture was then heated at 90°C for 3 min and rapidly cooled. The RNA dissociated from the cDNA-oligotex-dT30 was removed by centrifugation. The precipitate (cDNA-oligotex-dT30 complex) was washed twice with 200 μl of TE buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA) by centrifugation.

(b) Preparation of subtractive mRNA. The cDNA-oligotex-dT30 was dissolved in 100 μl of TE buffer containing 100 μg of $(dA)_{30}(dG)_{10}$ oligodeoxynucleotide. The suspension was heated at 65°C for 5 min, 11 μl of 5M NaCl was added and the incubation was continued at 37°C for 10 min to cover the free oligo(dT) residues on oligotex-dT30. The $(dA)_{30}(dG)_{10}$ not annealed to oligo(dT) was collected by centrifugation and kept at 4°C for reutilization. The precipitate (cDNA-oligotex-dT30) was dissolved in 200 μl of 1.25× hybridization buffer (12.5 mM Tris-HCl (pH 7.5), 125 mM NaCl, 1.25 mM EDTA, 0.125% SDS) containing 2 μg of $oligo(dT)_{12-18}$ to prevent mRNA from annealing to the remaining free oligo(dT) residues and 50 μl of H_2O containing 1 μg of poly $(A)^+$ RNA prepared from untreated cell (cell type B) was added. The hybridization reaction was performed at 55°C for 20 min and the reaction mixture was centrifuged at room temperature for 10 min. The supernatant (the first subtractive mRNA) was collected and stored at 4°C. To dissociate the hybridized mRNA from the cDNA-oligotex-dT30, the precipitate was dissolved in 400 μl of TE buffer, heated at 94°C for 3 min and rapidly cooled. The mixture was centrifuged and the supernatant was removed. The precipitate (cDNA-oligotex-dT30) was washed with 200 μl of TE buffer by centrifugation and dissolved in the $(dA)_{30}(dG)_{10}$ supernatant previously stored and incubated at 37°C for 10 min. The free $(dA)_{30}(dG)_{10}$ was again collected by centrifugation. The precipitate was dissolved in the first subtractive mRNA fraction and the second hybridization was performed at 55°C for 20 min. This subtractive hybridization was repeated four times in total.

(c) Amplification of subtractive mRNA by PCR. The subtractive mRNA specific to undifferentiated NEC14 cells (cell type B) finally obtained was annealed to new oligotex-dT30. 10 μl of 5% (w/v) oligotex-dT30 was added to the subtractive mRNA fraction and heated at 70°C for 5 min followed by rapid cooling in ice water. After addition of an equal volume of 2× TMK buffer, the mixture was incubated at 37°C for 20 min and centrifuged at 15000 rpm for 10 min at room temperature. The precipitate (mRNA-oligotex-dT30 complex) was dissolved in 250 μl of RT buffer containing 180 U of RNase inhibitor and 600 U of AMV reverse transcriptase. The reaction was carried out at 37°C for 1.5 h. The reaction mixture was then heated at 90°C for 3 min and rapidly cooled. The RNA dissociated from the cDNA-oligotex-dT30 complex was washed twice with 200 μl of H_2O by centrifugation and an oligo(dC) tail was added to the 3' end of the cDNA essentially according to Okayama and Berg (15). The precipitated was dissolved in 100 μl of TdT buffer (100 mM sodium cacodylate (pH 7.2), 2 mM $MnCl_2$, 0.1 mM DTT) containing 1 mM dCTP and 100 U of terminal deoxynucleotidyl transferase. The reaction was carried out at 37°C for 5 min and terminated by the addition of 20 μl 250 mM EDTA. The oligo(dC)-tailed cDNA-oligotex-dT30 was washed with H_2O several times by centrifugation. The cDNA linked to oligotex-dT30 was amplified by PCR. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM each of dNTPs, 1 μg of HindIII-$(dG)_{15}$ primer, 1 μg of EcoRI-$(dT)_{30}$ primer and 5 U of Taq polymerase (Ampli Taq; Takara Co.,Ltd.) (16). The reaction was carried out at 94°C for 1.5 min, 55°C for 2 min and 72°C for 3 min for 40 cycles. After 10 cycle reactions, the cDNA-oligotex-dT30 was removed from the reaction mixture by centrifugation owing to the instability of Latex particles to long period at high temperature and 5 U of Taq polymerase was added to the remaining reaction mixture. The primers, HindIII-$(dG)_{15}$, and EcoRI-$(dT)_{30}$ represent the oligodeoxynucleotides 5'GCGAAAGCTTGGG-GGGGGGGGGGG3' and 5'CGAGGAATTCTTTTTTTTT-TTTTTTTTTTTTTTTTTTTT 3', respectively.

(d) cDNA cloning in plasmid vectors. Ten mg of the vector DNA (pBluescript II KS^-) was added to the amplified cDNA and treated with *Eco*RI and *Hin*dIII at 37°C for 5 h. Small DNA fragments generated were removed by a gel filtration. The reaction mixture (200μl) was applied to a Sephacryl S-400 (Pharmacia Co., Ltd.) spin column made in 5 ml syringe (column volume, 5ml) and the cDNA was eluted together with the vector DNA by centrifugation at 1500 rpm for 3 min. Under these

conditions, only the cDNA longer than 250 nucleotides was eluted. The cDNA was ligated into the cloning site of the vector DNA with T4 DNA ligase at 15°C for 12 h and transfected into *E.coli* JM109 according to Inoue *et. al.* (17). The transformants developed in the LB-broth containing 25 μl/ml of ampicillin were stored at −80°C after addition of 7% DMSO.

(e) Comment. The subtractive cDNA can be cloned into a variety of convenient vector using an appropriate restriction sequence linked to the primers. The primers of oligo $(dG)_{15}$ and oligo$(dT)_{30}$ with the octanucleotide recognition sequence, e.g. for *Sse*8387I (Takara Co., Ltd.) would be useful to avoid a possible cleavage inside the cDNA sequence by *Eco*RI or *Hin* dIII. Linkage with a hexanucleotide recognition sequence such as that for *Xho* I is also recommended because of its presence in natural DNA sequences at relatively low frequency.

Differential colony hybridization

Poly $(A)^+$ RNAs prepared from undiffrentiated and HMBA-treated NEC14 cells as stated above were used as templates for the synthesis of ^{32}P-labeled cDNA pbobes with AMV reverse transcriptase. A typical reaction mixture contained 2 μg of poly $(A)^+$ RNA that had been pretreated at 65°C for 3 min, 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 20 mM DTT, 30 μM each of dNTPs, 4 μg of oligo$(dT)_{12-18}$ primer, 10 mg of Actinomycin D, 500 μCi each of [α-^{32}P]-dNTPs (3000 Ci/m mol; Amersham), 240 U of RNase inhibitor, and 100 U of reverse transcriptase in a final volume of 300 μl. The mixture was incubated at 37°C for 1.5 h and the reaction was terminated by the addition of 15 ml of 2N NaOH and heating at 70°C for 2 min. The reaction mixture was then neutralized and run over a Sephadex G-50 column to remove free nucleotides. ^{32}P-labeled cDNA probes were used to screen replica filters as shown in Fig.3. Colonies were replica plated onto 137 mm nylon filters, incubated overnight at 37°C on LB agar plates containing 25 μg/ml of ampicillin, and then on other plates containing 170 μg/ml of chloramphenicol overnight to amplify the plasmid. Colonies were then lysed and denatured (18). The filters were then prehybridized in 50% formamide containing 1M NaCl and 1% SDS at 42°C for 6 h and then hybridized in the same solution containing 100 μg/ml of denatured herring sperm DNA and 5×10^6 cpm/ml of ^{32}P-labeled cDNA probe at 42°C for 24 h. The filters were washed twice with 2× SSC containing 0.1% SDS at 65°C for 30 min and exposed to X-ray film with an intensifying screen at −80°C for 5 days.

Dot and Northern blot hybridization

Total cellular RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction procedure (19). For RNA dot hybridization, 5 μg of RNA were spotted onto a nylon filter and hybridized with ^{32}P-labeled cDNA probes. For Northern blot analysis, 5 μg of poly $(A)^+$RNA isolated by annealing to oligo$(dT)_{30}$-Latex (9) or 20 μg of total cellular RNA were subjected to electrophoresis in a 1.1% agarose gel containing 1.1 M formaldehyde in 10 mM sodium phosphate buffer (pH7.4) and transferred onto the nylon filter. ^{32}P-labeled cDNA was prepared with the multi prime DNA labeling system (Amersham Co., Ltd. RPN 1640) (21).

Sequencing of cDNA clones and computer analysis

Both strands of cDNA were sequenced by the dideoxynucleotide chain termination method of Sanger *et, al.* (22). Overlapping subclones of cDNA were generated by the step wise deletion method (23). Nucleotide and amino acid sequences were stored and edited in a DNASIS computer (Hitashi Soft Engineering Co., Ltd.).

RESULTS

Construction of a subtractive cDNA library specific to undifferentiated human EC cells

The human EC cell line NEC14, derived from a testicular germ cell tumor consisted mainly of typical EC stem cells that were small, polygonal and formed densely packed clusters as shown in Fig. 1A (10). The cells can be induced to morphologically differentiate by the addition of 10^{-2} M HMBA for several days (Fig.1B) (11,12). During this process the pattern of gene expression was markedly changed. All the viral enhancer-promoters so far tested were unable to function efficiently in undifferentiated NEC14 cells, but the activities of the SV 40, adenovirus and RSV enhancers were greatly increased after differentiation (11). The level of N-*myc* expression which was the highest among several cellular oncogenes tested in the undifferentiated cells decreased steeply from 12 to 18 h after addition of HMBA, but returned to the original level after 24 h (24).

To investigate the regulatory mechanism of gene expression during the early stage of HMBA-induced NEC14 cell differentiation, a subtractive cDNA library was constructed with the mRNAs present in undifferentiated NEC14 cells but not in the HMBA-treated cells using oligo$(dT)_{30}$-Latex and PCR (Fig.1). A method was designed to enrich for specific mRNAs

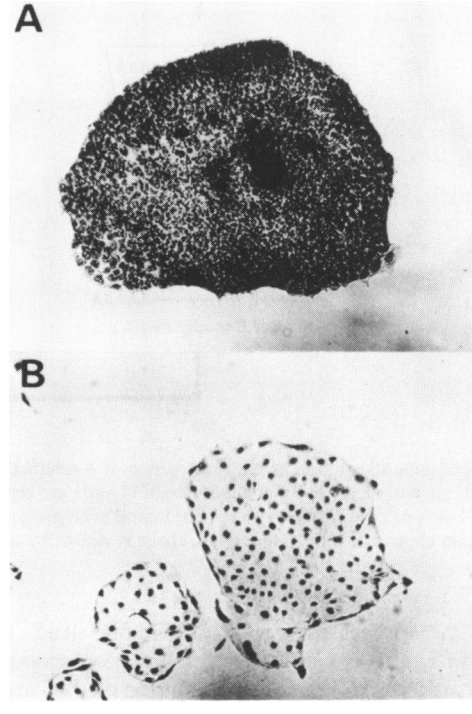

Figure 1. Colony morphology of NEC14 cells. A: Colony containing densely packed cells of stem cell morphology. B: After treatment of the cell with 10^{-2}M N,N'-hexiamethylane-bis-acetamide (HMBA) for 7 days, colony consists of flattened cells of differentiated morphology. Giemsa staining. ×40.

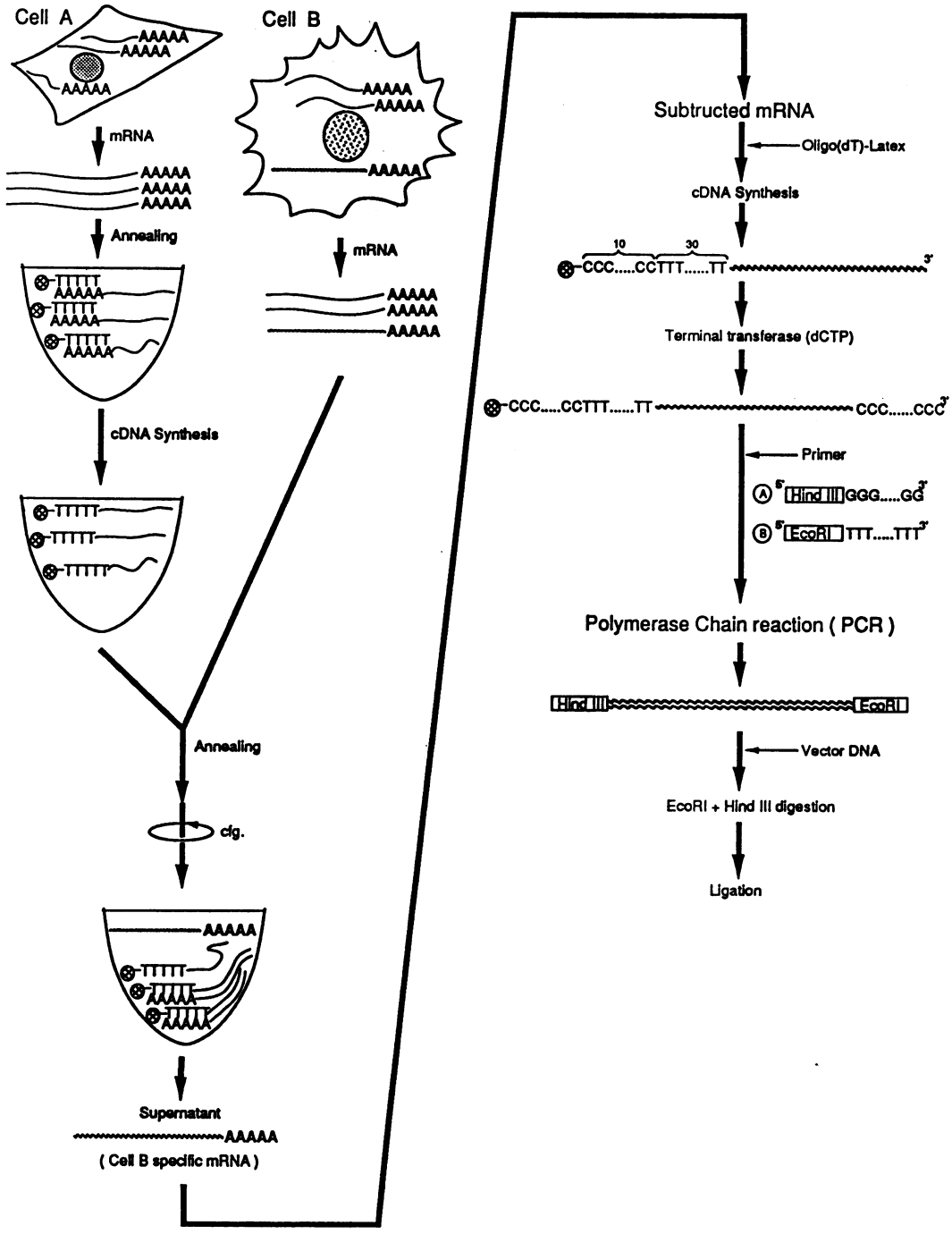

Figure 2. Schematic illustration of the construction of a subtractive cDNA library using oligo(dT)$_{30}$-Latex. The scheme for isolation of cDNA clones specific to cell type B is shown. Here, undifferentiated NEC14 cells are represented as cell type B and the HMBA-treated NEC14 cells are represented as cell type A. The oligo(dT)$_{30}$-Latex particle is shown by a shaded circle with protruding oligo(dT). The oligonocleotide, (dC)10(dT)$_{30}$ was linked to the Latex particle, but only chain is shown in an eppendorf tube. The cDNA chain is shown by a wavy line except the chain inside a tube.

avoiding the processes which inevitably lead to mRNA degradation and to isolate cDNA clones corresponding to low abundance mRNAs. Several features of the method are described below.

The mRNA prepared from the HMBA-treated NEC14 cells (cell type A) was annealed to oligo(dT)$_{30}$-Latex (Fig.2). The cDNA synthesis was efficiently carried out with AMV reverse transcriptase using the annealed mRNA as the primer. The addition of an excess amount of reverse transcriptase was essential to cover the surface of Latex particle completely, otherwise reverse transcriptase and thus presumably the cDNA chain, might stick to the Latex particle and cDNA synthesis would be hindered. The coating of Latex particles with BSA before reverse transcription was unsuccessful, presumably due to the presence of a trace amount of RNase even in electrophoretically purified preparation of BSA.

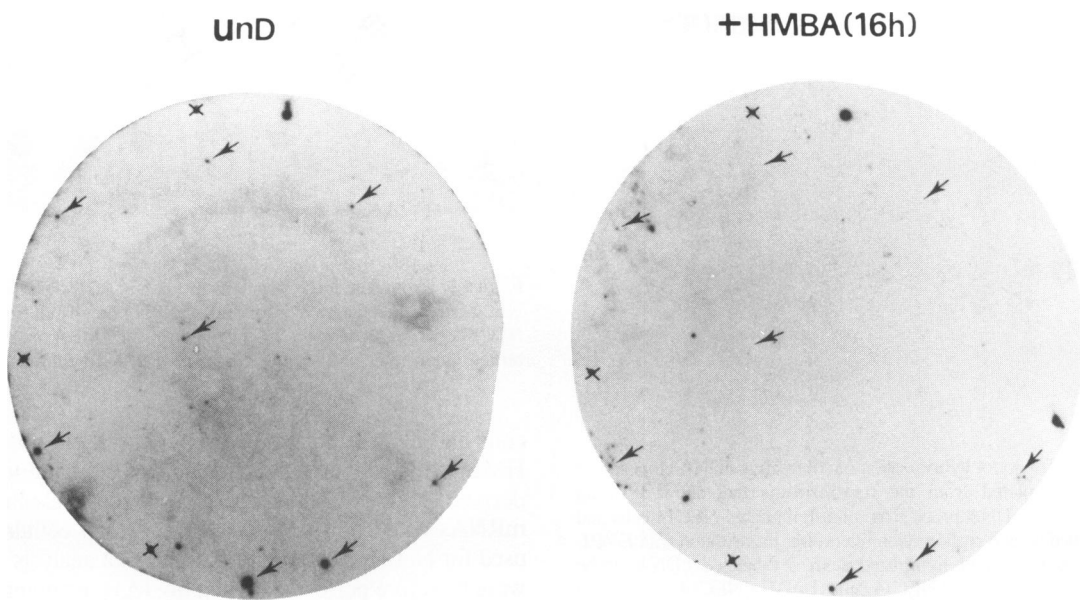

Figure 3. Screening of the subtractive cDNA library for clones preferentially expressed in undifferentiated NEC14 cells. Approximately 500 positive transformants were plated on a 137 mm-dish. Colonies were transferred to duplicate sets of nylon filters and hybridized with ^{32}P-labeled cDNA probe (1 to 3×10^9 cpm/μg) prepared from either undifferentiated NEC14 cells (UnD) or NEC14 cells treated with HMBA for 16 h (+HMBA, 16h) as described in Materials and Method. The arrows indicate the position of colonies preferentially hybridized to ^{32}P-labeled probe from undifferentiated NEC14 cells.

The enrichement of mRNAs present in undifferentiated NEC14 cells (cell type B in Fig.2) but not in the HMBA-treated cells (cell type A) was carried out by successive subtractive hybridization between oligo(dT)$_{30}$-Latex linked cDNA and mRNA. Unhybridized mRNA was collected by a brief centrifugation and rehybridized to the cDNA linked to oligo(dT)$_{30}$-Latex. Several cycles of this subtractive hybridization could be done in a relatively short time period and the subtractive mRNA could be enriched in a small volume of the unhybridized mRNA fraction much more efficiently than is possible with the usual adsorbent hydroxyapatite.

For amplification of the subtractive cDNA sequences by PCR, primers containing the recognition sequence of either *Eco*RI or *Hin*dIII were used (Fig.2). The restriction sequences linked to the primers can be changed for appropriate restriction enzymes depending on the cloning vector used. Attachment of *Xho*I linkers to both primers was also successful in amplification of cDNA sequences by PCR and for cloning of the cDNA into the cloning vector with calf intestine alkaline phosphatase-treated *Xho*I cohesive ends (data not shown). The *Xho*I sequence may be a preferable linker since its recognition sequence seems to appear at a relatively low frequency in natural DNA sequences. The attachment of an octanucleotide recognition sequence, e.g. for *Sse*8387I, might be useful to avoid possible cleavage inside the cDNA sequence by a restriction enzyme(s). Attachment of the *Not*I octanucleotide recognition sequence GCGGCCGC so far tested was unsuccessful in cDNA chain elongation presumably due to its GC rich sequence.

Characterization of amplified subtractive cDNA clones

The bacterial transformants were plated in the presence of isopropyl β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (x-gal) to estimate how many of the transformants contained cDNA inserts. The numbers of colonies that showed transparent and blue colors were nearly equal indicating that approximately 50% of the transformants contained cDNA inserts. To measure the length of each cDNA insert, 21 positive tranformants were picked at random and the plasmid DNAs isolated were subjected to electrophoresis after cleavage with *Eco*RI and *Hin*dIII. The lengths of cDNA ranged from 0.5 to about 5.0 kb. Two plasmids which did not generate a cDNA inserts may be an empty plasmid or contain a very short segment of cDNA. An average cDNA length of the plasmid was estimated to 1.7 kb.

Since the subtractive cDNA was amplified by PCR, it is possible that selected species of cDNA clones were preferentially amplified and that the cDNA library mainly consisted of these selected species of cDNA clones. To examine this posibility, about 200 plasmid DNAs were electrophoresed after cleavage with *Eco*RI and *Hin*dIII and the transformants that generated cDNA inserts of similar length were selected and grouped. The plasmid DNAs from these transformants were then cleaved with the four base recognition restriction enzyme *Taq*I and the fragments generated were compared in each group. Among a total of 23 clones, divided into 6 groups, tested, 3 pairs of cDNA inserts were estimated to have orignated from the same clone. These results suggest that approximately 80% of the positive transformants contain independent cDNA clone.

To estimate the efficiency of subtraction and to isolate the clones containing cDNA inserts specific to undifferentiated NEC14 cells, approximately 500 positive transformants were plated and transferred in duplicate to nylon filters. One of the filters was hybridized with the ^{32}P-labeled cDNA probe from undifferentiated NEC14 cells and the other with the ^{32}P-labeled cDNA from HMBA-treated NEC14 cells. To detect the cDNA clones that derived from low abundance mRNAs, ^{32}P-labeled cDNA probes with high specific activity were synthesized in the presence of 4 [α-^{32}P]dNTPs. After autoradiography, the intensity of the signal from each colony was compared between these two filters (Fig.3). The number of colonies which showed

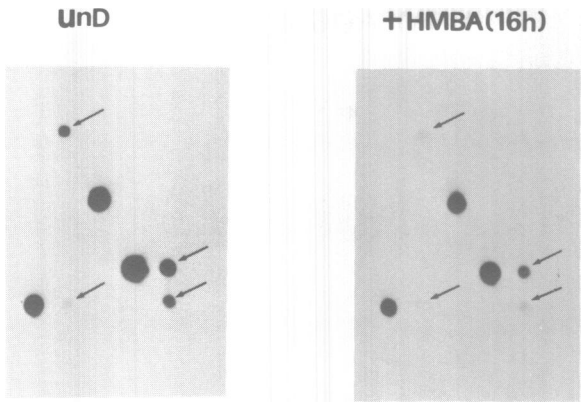

Figure 4. Differential DNA-dot hybridization of subtractive cDNA clones. The plasmid DNAs were isolated from the transformants that showed strong hybridization signal to the cDNA probe from undifferentiated NEC14 cells and 1 μg of each was spotted on two replica nylon filters after linearization with *Eco*RI. The DNAs were denatured and hybridized with ^{32}P-labeled cDNA probe prepared from either undifferentiated NEC14 cells (UnD) or NEC14 cells treated with HMBA for 16 h (+HMBA, 16 h). The arrows indicated the clones that preferentially hybridized to the probe from undifferentiated NEC14 cells.

visible signals on both filters ranged from 100 to 250 depending on the specific activity of probes and exposure time. A fraction of positive dots with weak intensity on the X-ray film could not be seen on the photograph. Among these positive dots, approximately 15% of the dots showed stronger intensity with the probe prepared from undifferentiated NEC14 cells. Eight out of these colonies are indicated by the arrows. To confirm the preferential hybridization of these colonies to the undifferentiated cell probe, these colonies were picked and plasmid DNAs were isolated from 20 of them. Using these plasmid DNAs, the differential screening was performed by DNA dot hybridization. As shown in Fig.4, 4 out of 20 plasmid DNAs showed a stronger signal with the undifferentiated cell cDNA probe. As shown in the following section (Fig.5), all of these identified cDNA clones were hybridize preferentially to RNA prepared from the undifferentiated cells. These results suggest that approximately 3.0% (15/100×4/20×100) of the transformants in the subtractive cDNA library contain cDNA inserts specific to undifferentiated NEC14 cells. This value indicates an efficient enrichment of the cDNA sequences, since the efficiency of identification of differentially expressed clones derived from moderately abundant mRNA species is usually around 0.01% when the library is constructed without the enrichment process.

Repression of the expression of undifferentiated cell-specific gene after treatment of NEC14 cells with HMBA

Alteration in the expression levels of differentially expressed genes in NEC14 cells after induction of differentiation by the addition of HMBA was followed by Northern blot hybridization with total or poly (A)-containing RNA. First, the expression levels of these genes were compared by RNA dot hybridization (Fig.5). Total cellular RNAs prepared from undifferentiated NEC14 cells and from cells treated with HMBA for 16 h were spotted on the filter and hybridized with ^{32}P-labeled probes of 9 subtractive cDNA clones. All of these cDNA clones were hybridized preferentially with the undifferentiated cell RNA. Among them, the expression levels of clones, ECX-2, ECA-10, ECA-17 and EC4−50, were very low even in the undifferentiated

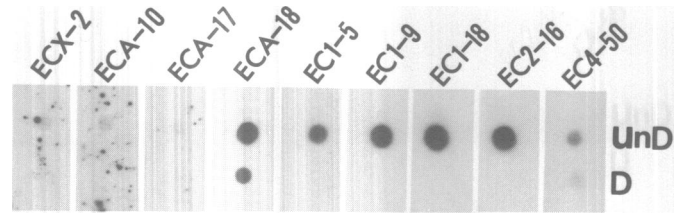

Figure 5. Expression levels of subtractive clones in HMBA-treated and untreated NEC14 cells. Five μg each of total cellular RNAs prepared from undifferentiated NEC14 cells (UnD) and NEC14 cells treated with HMBA for 16 h (D) was spotted on the nylon filter and hybridized with ^{32}P-labeled subtractive cDNA clones.

cells and hybridization signals were scarcely observed with the HMBA-treated cell RNA, indicating that these clones were derived from low abundance mRNAs. These low abundance mRNAs could not be detected when total cellular RNAs were used for Northern blotting. Northern blot analysis of these clones were therefore performed with poly (A)-containing RNAs except clone EC1−18 (d) which showed an abundant expression in Fig.5. RNAs were prepared from the HMBA-treated cells at 6 h intervals. As shown in Fig.6, the expression of clone EC4−50 (c) was repressed shortly after treatment with HMBA and no transcript was detected after 6 h. The expression levels of clones ECX-2 (a) and ECA-17 (b) decreased slightly later than that of EC4−50 and residual levels expression were observed after 6 h. The expression levels of clones EC1−18 (d) and ECA-10 (e)were unchanged until 6 h but decreased steeply thereafter. The lengths of ECA-17 and EC4−50 transcripts were larger than that of 28 S ribosomal RNA (4.7 kb) and the length of ECX-2, EC1−18 and ECA-10 transcripts were between those of 28 S and 18 S (1.9 kb) ribosomal RNAs. The lengths of all the isolated cDNA clones were smaller than those of their transcripts ranging from 0.5 to 2.5 kb. We are currently isolated full-length cDNA for these clones in the subtractive cDNA library by PCR using the *Hin*dIII-(dG)15 and the sequence inside the cDNA fragment as primers.

Computer analysis of the sequences with the data available through Genebank and EMBL databanks revealed that clone EC2−16 has high homology with the C-terminal portion of the human mitochondorial creatine kinase gene (25) and clone EC1−5 with the human testis specific protein kinase C (26). All the cDNA clones of low abundance mRNAs showed no homology with any known gene sequences except clone ECX-2 which has 73% homology with the 3′-noncoding 400 bp sequence of mouse Id (inhibitor of DNA binding) gene (27).

DISCUSSION

The present method for construction of a subtractive cDNA library has the following features. (a) A single strand cDNA was synthesized from mRNA using the oligo(dT)$_{30}$ primer covalently linked to Latex particle (9) so that after subtractive hybridization unhybridized mRNA could be easily separated from the cDNA-mRNA hybrid by a brief centrifugation. Four cycles of this subtractive hybridization effectively enriched the subtracted mRNA specific to the undifferentiated human EC cell line, NEC14. (b) The single strand cDNA made from the subtractive mRNA was amplified by PCR to isolated cDNA clones derived from low abundance mRNAs. The restriction analysis of the

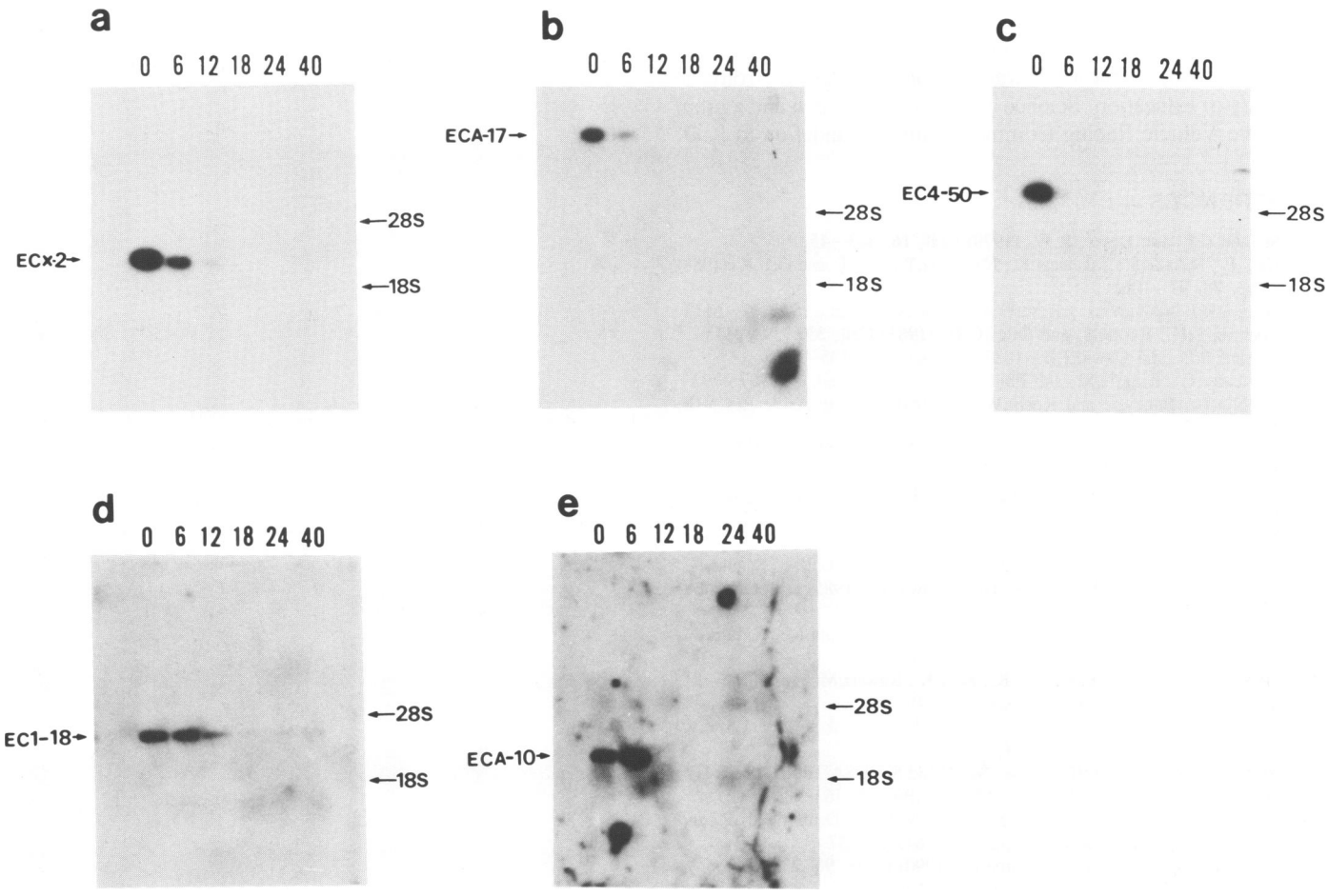

Figure 6. Alteration in expression levels of subtractive clones in NEC14 cells after induction of differentiation. Total cellular RNA or poly A-containing RNA was prepared from NEC14 cells after treatments with 10^{-2}M HMBA at the times indicated. Five μg of poly A-containing RNA (a,b,c,e) or 20 μg of total cellular RNA (d) were electrophoresed, transferred to nylon filters and hybridized with 3×10^6 cpm/ml of ^{32}P-labeled subtractive cDNA clones with specific activities of 10^9 cpm/mg.

amplified subtractive cDNA clones by cleavage with the tetranucleotide recognition endonuclease *Taq*I suggested that at least 80% of the transformants in the library contained independent cDNA clones. The results indicate that the fraction of cDNA clones which was preferentially amplified during PCR was minor, although smaller molecules seems to be more efficiently amplified than larger molecules (28). (c) The subtractive cDNA can be cloned into a convenient vector using primers containing an appropriate restriction sequence. The subtractive cDNA library was subsequently screened for cDNA clones specific to the undiffrentiated EC cells by colony hybridization and DNA dot hybridization. All of the cDNA clones thus selected were hybridized preferentially to RNA prepared from the undifferentiated cells. These results suggest that approximately 3% of the transformants in the cDNA library contain cDNA inserts specific to the undifferentiated cells. The cDNA clones corresponding to low abundance mRNAs showed a very weak signal on the autoradiogram even with the cDNA probe prepared from undifferentiated cells. A cDNA probe prepared with 4 [α-^{32}P]dNTPs was helpful in identifying these clones.

The lengths of the subtractive cDNA clones specific to undifferentiated NEC14 cells ranged from 0.5 to 2.5 kb and were shorter than those of their corresponding mRNAs. Full length cDNA of these clones could however, be isolated by PCR. Since the oligo(dC) tail was added to 3′ end of the cDNA, the full length cDNA can be screened in the subtractive cDNA library by PCR using the oligo(dG)$_{15}$ as the upstream primer and the internal sequence of the cDNA fragment as the downstream primer. Using this method we could isolated a full length cDNA for the mouse Id gene (27) in a subtractive cDNA library similarly constructed from undifferentiated mouse F9, EC cells (unpublished data).

To enrich full length cDNA, we are currently constructing a library from the subtractive sense strand of cDNA. In this method, two types of mRNA from the undifferentiated and HMBA-treated NEC14 cells were converted to cDNA immediately after preparation using the oligo(dT)$_{30}$-Latex. The sense strand of cDNAs from the undifferentiated cells was synthesized from the cDNA-oligo(dT)$_{30}$-Latex by two cycles of PCR using only the oligo(dG)$_{15}$-HindIII primer. The subtractive hybridization was then carried out between the cDNA-oligo(dT)$_{30}$-Latex made from the HMBA-treated cell mRNA and the sense strand of cDNA synthesized by PCR.

Analysis of the specific cDNA clones isolated from the undifferentiated NEC14 cells by Northern blotting indicated that the expression levels of multiple genes decreased steeply shortly after induction of differentiation by the addition of HMBA. The decrease occurred sequentially in a time-dependent manner.

ACKNOWLEDGMENTS

We thank Dr. Yasuhiro Furuichi and Dr. Hiroto Okayama for valuable advice. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and a grant from the Vehicle Racing Commemorative Foundation to K.O.

REFERENCES

1. St.John,T.P. and Davis, R.W. (1979) Cell, **16**, 443–452.
2. Hara,E., Nakada,S., Takehana,K., Nakajima,T., Iino,T. and Oda,K. (1988) Gene, **70**, 97–106.
3. Weigel,R.J. and Nevins, J.R. (1990) Nucleic Acids Res., **18**, 6107–6112.
4. Cochran,B.H., Rettle,A. and Stiles,C.D. (1983) Cell, **33**, 939–947.
5. Sargent,T.D. and Dawid,I.B. (1983) Science, **222**, 135–139.
6. Schneider,C., King,R.M. and Philipson, L. (1988) Cell, **54**, 787–793.
7. Timblin,C., Battey,J. and Kuehl,W.M. (1990) Nucleic Acide Res., **18**, 1587–1593.
8. Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlichi,H.A. and Arnheim, N. (1985) Science, **230**, 1350–1354.
9. Kuribayashi,K., Hikata,M., Hiraoka,O., Miyamoto,C. and Furuichi,Y. (1988) Nucleic
Acids Res. Symposium Series **19**, 61–64.
10. Sekiya,S, Kawata,M., Iwasaki,H., Inaba.N., Sugita,M., Suzuki,N., Motoyama,T., Yamamoto,T. and Takamizawa, H. (1985) Differentiation, **29**, 259–267.
11. Hasegawa,T., Nakada,S., Nakajima,T., Oda,K., Kawata,M., Kimura,H. and Sekiya,S. (1990) Differentiation **42**, 191–198.
12. Sekiya.S., Kimura,H., Yamazawa,K., Kera,K., Kawata,M., Takamizawa,H. and Oda, K. (1990) Genecol Oncol., **36**, 69–78.
13. Okayama,H., Kawaichi,M., Brownstein, M., Lee,F., Yakota,T. and Arai,K. (1987) Method in Enzymol., **154**, 3–28.
14. Aviv,H. and Leder,P. (1972) Proc. Natl.Acad.Sci.USA., **69**, 1403–1412.
15. Okayama,H. and Berg, P. (1982) Mol.Cell.Biol. **2**, 161–170.
16. Lawyer, f.C. Stoffel,S., Saiki,R.K., Myambo,K.B., Drummond ,R. and Gelfand, D.H. (1989) J.Biol.Chem., **264**, 6427–6437.
17. Inoue,H., Nojima,H. and Okayama,H. (1990) Gene, **96**, 23–28.
18. Thayer,R.E. (1979) Anal. Biochem., **98**, 60–63.
19. Chomczynski,P. and Sacchi,N. (1987) Anal.Biochem., **162**, 156–159.
20. Oda,K., Masuda-Murata, M., Shiroki,K. and Handa,H. (1986) J.Virol., **58**, 125–133.
21. Feinberg,A.P. and Vogelstein,B. (1983) Anal.Biochem., **132**, 6–13.
22. Sanger,F., Nicklen,S. and Coulson, A.R. (1977) Proc.Natl.Acad.Sci.USA., **74**, 5463–5467.
23. Yanischi-Perron, C., Vieira,J. and Messing,J. (1985) Gene, **33**, 103–119.
24. Hasegawa,T., Hara,E., Takehana,K., Nakada,S., Oda,K., Kawata,M.,Kimura,H. and Sekiya.S. (1991) Differentiation, **47**, 107–117.
25. Haas, R.C. and Stranss,A.W. (1990) J.Biol.Chem., **265**, 6921–6927.
26. Beebe,S.J., Oyen,O., Sandberg.M., Froysa,A., Hansson,V. and Jahnsen,T. (1990) Mol.Endocrinol. **4**, 465–475.
27. Benezra,R., Davis,R.L., Lockshon,D., Turner,D.L. and Weintraub,H. (1990) Cell, **61**, 49–59.
28. Jeffreys,A.J., Wilson,V., Neumann,R. and Keyte,J. (1988) Nucleic Acids Res., **16**, 10953–10971.