Application for PCR technology to subtractive cDNA cloning: identification of genes expressed specifically in murine plasmacytoma cells

Cynthia Timblin, James Battey[1] and W.Michael Kuehl
NCI-Navy Medical Oncology Branch, National Cancer Institute, National Institutes of Health and Naval Hospital, Bethesda, MD 2084-5015 and [1]Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892, USA

Received October 11, 1989; Revised and Accepted February 21, 1990

ABSTRACT

We describe a simple method for preparing a renewable source of subtractive cDNA which can be used as a hybridization probe or as insert which can be cloned into a variety of convenient vectors. This has been done by ligating a double-stranded oligonucleotide to each end of double-stranded subtractive cDNA, and then using this oligonucleotide sequence to amplify the heterogeneous population of cDNA molecules using the polymerase chain reaction and thermostable Taq DNA polymerase. This method improves the chances for identifying cDNA clones representing low abundance mRNAs that are expressed differentially. Using this approach, we have identified cDNA clones which detect three different low abundance mRNAs that are expressed in mouse plasmacytoma cell lines but not in mouse pre-B or B lymphoma cell lines.

INTRODUCTION

Without a specific probe for hybridization or a specific bioassay, identification of low abundance and rare mRNAs which are expressed in a cell specific manner continues to be a vexing problem. Differential screening of cDNA libraries with labelled first strand cDNAs synthesized from unfractionated RNA can detect clones representing highly abundant mRNA species (i.e. about 0.1% or more of mRNA). Preparation of subtractive cDNA probes permits the identification of differentially expressed clones which represent moderately abundant species (i.e about 0.01% or more of mRNA), although prior fractionation of mRNA by size or subcellular localization may enhance the sensitivity of either approach(1,2). A number of laboratories have prepared subtractive cDNA libraries so that fewer clones need to be screened(3−7). Despite a number of spectacular successes using subtractive cDNA technology(4,8,9), this approach continues to be technically difficult. In this report, we present a novel strategy for preparing subtractive cDNA probes and subtractive cDNA libraries, by incorporating PCR technology into these procedures. We have applied this approach to identify cDNA clones from mRNAs which are expressed in a mouse plasmacytoma cell but not in a highly differentiated murine B lymphoma cell line.

MATERIALS AND METHODS

Cells. The 4N2.1 and 653FB cell lines are immunoglobulin expression variants derived from MPC 11 and MOPC 21 mouse plasmacytomas, respectively(10,11). The A20.2J B lymphoma cell line expresses high levels of surface Ia antigen, and also expresses and secretes low levels of IgG2a(12). The 315P and 315J cell lines were obtained from Richard Lynch. The S107, J558, and J558L cell lines were obtained from Sherrie Morrison. All other cell lines were obtained from the ATCC unless described previously (18). Cells were grown in Petri dishes or roller bottles in RPMI 1640 supplemented with 5% fetal calf serum, 50 μM mercaptoethanol, penicillin, and streptomycin.

Cytoplasmic RNA isolation. A post-nuclear supernatant was prepared from cells which had been lysed with 0.5% Nonidet P-40(13). In some instances three volumes of a 4 M guanidine thiocyanate solution were added to the supernatant, and RNA was purified by centrifugation through CsCl gradients(14). Alternatively RNA was purified by phenol-chloroform extraction in the presence of 1% SDS, followed by ethanol precipitation, but was then centrifuged through 5.7 M CsCl to remove DNA. In both cases the RNA was further purified by precipitation with 2 M LiCl(15). Poly(A)+ RNA was selected by one cycle of oligo(dT)-cellulose chromatography(16). The exception was RNAs used as template for first strand cDNA synthesis, all of which were selected by two cycles of oligo(dT)-cellulose chromatography.

RNA analysis. Cytoplasmic RNA was fractionated on 1% agarose gels containing 0.22 M formaldehyde, and transferred to nitrocellulose filters(17). The filters were hybridized, washed, and exposed to XAR-5 film at −70° C with intensifying screens as described previously(18).

First strand cDNA synthesis. For preparative reactions, ten ug of mouse plasmacytoma 4N2.1 cell line poly(A)+ cytoplasmic RNA which had been twice selected on oligo(dT)-cellulose was heated to 70° C for 3 min. and placed on ice. After addition of each dNTP to 500μM,100μCi of α-^{32}P-dCTP(3000 C_i/mmole), 10μg of random hexamer primer(Pharmacia), 80 U of RNAsin(Promega), 10μg of nuclease-free bovine serum

albumin, and 1000 U of MMLV reverse transciptase (BRL), the 100 μl reaction in reverse transcriptase buffer (BRL) was incubated for 60 minutes at 37° C. Five individual reactions were pooled, extracted with phenol-chloroform, hydrolyzed in 0.1 N NaOH at 70° C for 20 min., adjusted to 100 mM Tris, neutralized by addition of HCl, and fractionated on a Sephadex G-50 column. The excluded fractions were pooled and ethanol precipitated(19).

Hybridizations. Hybridizations were done in 700 μl microfuge tubes in 5−50 μl of hybridization buffer containing 20 mM Tris-HCl(pH 7.7), 600 mM NaCl, 2 mM EDTA, 0.2% SDS, and 2−5 μg/μl of poly(A)+ RNA. The samples were overlaid with 100 μl of mineral oil, boiled for 5 minutes, and hybridized at 70° C for 1−2 days to reach a Rot (corrected to standard salt conditions) of greater than 3000(20).

Hydroxyapatite(HAP) chromatography. All steps were done at 60° C essentially as described by Alt et al(2). Hybridized samples were diluted to 1 ml with 0.08 M $NaPO_4$(pH 6.8)-150 mM NaCl, mixed three times with 0.5 g of HAP, poured into a column, eluted with six 1 ml aliquots of 0.10 M $NaPO_4$-150 mM NaCl, and then with six 1 ml aliquots of 0.5 M $NaPO_4$-150 mM NaCl. The material in the 0.08−0.1 M $NaPO_4$ fractions (i.e. single stranded) in some cases was rechromatographed on a second HAP column. Similarly, the material eluted in the 0.5 M NaPO4 fractions (i.e. double-stranded) was desalted, concentrated, and also rechromatographed on a second HAP column. To desalt and concentrate HAP eluates, pooled samples were chromatographed on NENSORB 20 (DuPont), eluted with 50% ethanol, and dried under vacuum(19).

Second strand cDNA synthesis. The positively selected RNA-cDNA heteroduplex was converted to double-stranded cDNA by incubation for 5 hours at 14° C in 40 μl containing 20 mM Tris-HCl(pH 7.5), 10 mM $MgCl_2$, 10 mM $(NH4)_2SO_4$, 100 mM KCl, 312 μM of each dNTP, 1 μg bovine serum albumin, 2 U of RNaseH(BRL) and 10 U of DNA polymerase I (BRL)(19). The material was then extracted with phenol- chloroform, separated on Sephadex G-50, the excluded fractions concentrated to 100 μl on a speed-vac, and then precipitated with ethanol.

Polishing, kinasing, and linker ligation. The double-stranded cDNA was incubated for 30 minutes at 37° C in 60 μl containing T4 DNA polymerase buffer (70mM Tris-HCl,pH 7.4, 10 mM $MgCl_2$, 5 mM DTT), 420 uM of each dNTP, 500 uM ATP, 10 U of T4 DNA polymerase, and 20 U of T4 polynucleotide kinase(19). The resultant products were extracted with phenol-chloroform and chloroform, and precipitated with 2 volumes of ethanol after addition of 10 μg of yeast tRNA and sodium acetate to 0.3 M. The sample was then ligated overnight at 14° C in 10 μl of T4 ligase buffer containing 120 ng of the amplification linker (see Fig.1),and 2 U of T4 DNA ligase(BRL). Following phenol-chloroform extraction, fragments of DNA greater than approximately 200 bp were separated from excess linkers and smaller DNA fragments by Sepharose 4B chromatography in a buffer containing 20 mM Tris-HCl (pH 7.6), 0.1 M NaCl, and 1 mM EDTA. The column was calibrated by chromatography of φX174 Hae III markers. The appropriate fractions were pooled and precipitated with ethanol after addition of 10 μg of yeast tRNA and sodium acetate to 0.25 M. The precipitate was collected, washed with 80% ethanol, and resuspended in 20 μl of water.

Polymerase chain reactions. PCR was performed in 100 μl volumes in a Perkin-Elmer/Cetus Thermal Cycler, using Taq polymerase and reaction conditions provided by Perkin-Elmer/Cetus. Aliquots of the subtracted insert were amplified or reamplified by addition of 1 μg of the amplification oligonucleotide (see Fig.1), using 30 cycles of 1 minute at 94° C, 2 minutes at 37° C, and 5 minutes at 72° C. When necessary, one tenth of the amplified insert was reamplified in 100 μl of a fresh reaction mixture for one cycle, and then chased by three additional cycles which omitted the 94° C incubation. Inserts were obtained from λgt10 phage using 0.5 μg each of phage specific oligonucleotides (see below) and the same PCR program.

Preparation of λgt10 subtractive cDNA library. Approximately 1 μg of amplified subtractive insert derived from a chase reaction (see above) was digested with SalI to remove the amplification linkers. The SalI sites were converted to EcoRI sites using SalI/EcoRI adapters(NEB), and the product fractionated on a 5% polyacrylamide gel. Fragments of DNA greater than 200 bp were electroeluted and then concentrated by passage through an Elutip-d minicolumn. The resultant insert was ligated to Eco RI-cut, dephosphorylated λgt10(Stratagene), packaged, and plated, yielding 1.2 million plaques(19).

Isolation of λgt10 inserts. A plug of agarose containing a fresh phage plaque was removed with a Pasteur pipette, resuspended in 200 μl of SM buffer(500 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin), and mixed for two hours at room temperature. The resultant supernatant was stored at 4° C as a phage stock, but 20−50 μl was boiled for 5 minutes, and then subjected to 30−50 cycles of PCR amplification as described above, using an appropriate pair of oligonucleotides derived from left(−) and right(+) sides of the λgt10 EcoRI cloning site (position 0), i.e. pair A = (−29)AAGTTCAGCCTGGTTAA-GTCCAAG and (+29)TATTTCTTCCAGGGTAAAAAGC; and pair B = (−75) TTTCGAGCTGCTCTATAGACTGCT and (+84)CAAATACAGTTTTTCTTGTGAAGATT. The amplified fragment was fractionated on a 1.5% agarose gel, and isolated using a Gene Clean kit (Bio101).

Labelling of probes. Five to 25 ng of DNA were labelled with α-^{32}P-dCTP using a random priming labelling kit (BRL). The DNA used for labelling included purified DNA fragments, random primed first strand cDNA, and PCR amplified subtractive insert.

Screening λgt10 cDNA library. Plaques were picked onto ten duplicate 129 position grids on 150 mm dishes. Two nitrocellulose lifts were made from one of the two plates. These lifts were processed, hybridized, washed, and residual probe stipped off using standard techniques(19). Phage was picked from the second plate and replaqued before isolation of insert by PCR.

RESULTS

Mouse plasmacytoma cell lines differ from a highly differentiated, IgG2a immunoglobulin secreting B lymphoma cell line called A20.2J by a number of notable features: 1) the distinctive plasma cell morphology including extensive and well-developed rough endoplasmic reticulum and Golgi apparatus(21,22); 2) the lack of expression of certain B cell surface markers (e.g. Ia antigens)(23); and 3) the lack of significant c-myc mRNA

TABLE I. Fractionation of 4N2.1 Plasmacytoma cDNA by Subtraction and Positive Selection

		Yield, ng (%)
First strand cDNA		15,000 (100)
50 μg 4N2.1 poly(A)+RNA		
Subtraction #1		
250 μg A20.2J poly(A)+RNA		
	HAP 1a 14% ss	1755 (11.7)
	HAP 1b 62% ss	870 (5.8)
Subtraction #2		
250 μg A20.2J poly(A)+RNA		
	HAP 2a 64% ss	510 (3.4)
Positive selection		
250 μg 4N2.1 poly(A)+RNA		
	HAP 3a 38% ds	150 (1.0)
	HAP 3b 83% ds	75 (0.5)
*Sepharose 4B	<200 bp 90%	21 (0.14)
	>200 bp 10%	2 (.015)

*50% of material accidentally lost just before this step

expression from the normal allele (24). The 4N2.1 variant plasmacytoma cell line does not express detectable immunoglobulin heavy chain mRNA and has deleted the functional kappa light chain gene, both of which are expressed in the parental MPC 11 plasmacytoma cell line(10). However, 4N2.1 continues to express the aberrant kappa light chain constant region fragment, which comprises about 1% of the mRNA present in these cells. The A20.2J B lymphoma line expresses and secretes IgG2a and kappa light chain, the latter comprising about 0.1% of the mRNA in this cell line(12). By Northern blot analysis, we found that 4N2.1 and A20.2J cells express similar amounts of J chain, intracisternal A particle, and PC.1 antigen mRNAs (C.Timblin and M. Kuehl, unpublished). We were interested in identifying genes that are expressed specifically in terminally differentiated plasmacytoma cells. At the outset, however, we knew of no genes that are expressed uniquely in MPC 11 and other mouse plasmacytoma cells but not in A20.2J and other B cell lines.

We expected to recover only a very small fraction of cDNA after the multiple steps required for preparation of subtractive insert, and thus used fifty μg of twice oligo(dT)-cellulose selected poly(A)+ cytoplasmic RNA from 4N2.1 cells to prepare first strand cDNA. Since the presence of actinomycin D inhibits synthesis of first strand syntheis by MMLV reverse transciptase approximately twofold, we omitted actinomycin D from this reaction. Random hexamer rather than oligo(dT) was used as primer so that we could obtain a more comprehensive cDNA representation of the plasmacytoma mRNA. Fifteen μg (i.e. a 30% yield) of first strand cDNA, with an average size greater than 1.5 kb based on migration on a polyacrylamide gel, was obtained in this initial reaction. Table I shows a flow chart of the subsequent fractionation of the first strand cDNA.

First, the cDNA was hybridized to 250 μg of A20.2J poly(A)+ cytoplasmic RNA to a Rot of 4000. (since poly(A)+ cytoplasmic RNA which has been purified by only one cycle of oligo(dT)-cellulose chromatography is estimated by us to be approximately 50% pure(16), the Rot corrected for pure poly(A)+ RNA is about 2000). Hydroxyapatite(HAP) chromatography of the hybridized material removed 86% of the first strand cDNA in the double-stranded fraction (by contrast, following a self-annealing hybridization, only 15% of the first strand cDNA was localized in the double-stranded fraction). After a second HAP column, the single-stranded cDNA was subjected to an identical subtraction and a single HAP column prior to a positive selection and two additional HAP columns to generate cDNA:RNA duplex (Table I). Based on the HAP fractionation of cDNA into single-stranded (ss) and double-stranded (ds) fractions for each of these various steps, the overall calculated yield of first strand cDNA as RNA:cDNA duplex after two rounds of subtraction with A20.2J RNA and one round of positive selection with the 4N2.1 parental RNA was 1.75%. The actual yield was 0.50%, due to losses during desalting and concentration, incomplete elution from HAP columns, etc.

The RNA:cDNA duplex was converted to ds cDNA, which was treated with T4 DNA polymerase and T4 kinase to generate phosphorylated blunt termini. After these steps, less than 100 ng of first strand cDNA remained. To permit PCR amplification, we attached a double-stranded 'amplification linker' oligonucleotide (Fig. 1), which contained EcoRI, KpnI, and SalI restriction sites, to the two termini of the cDNA fragments. This synthetic oligonucleotide had a 5'-phosphate at the blunt end, and a non-phosphorylated 5' overhang at the other end, so that only the blunt end would efficiently ligate to itself or other blunt ended DNA molecules. Following ligation, the subtractive cDNA was chromatographed on Sepharose 4B to separate larger cDNA fragments from the smaller cDNA fragments and excess of ligated and unligated oligonucleotide linkers. Only 10% (2ng) of the first strand cDNA eluted in the fractions containing DNA fragments greater than 200 bp. These larger DNA fragments were then subjected to PCR amplification for 30 cycles with an appropriate amplification oligonucleotide primer (Fig. 1).

Analysis of the amplified DNA on non-denaturing agarose and polyacrylamide gels demonstrated an apparent size discordance which indicated that most of the DNA was not completely double-stranded(25). This conclusion was confirmed by the observation that the majority of this material is sensitive to S1-nuclease digestion (J. Battey, unpublished). Because of the expected heterogenity of the subtractive cDNA, we suspect that most of the amplified DNA existed in partially single-stranded structures of types a and c as depicted in Fig. 1. In an attempt to convert the DNA to a fully duplex form, we used a PCR chase protocol as described in Materials and Methods. Based on the concordance of migration of this 'PCR chased' material on acrylamide and agarose gels, we concluded that most of the material existed in a fully duplex form following the PCR chase reaction. The high proportion of subtracted cDNA molecules less than 200 bp (Table I) and the absence of a significant number of inserts greater than 600 bp after selection on a polyacrylamide gel reflects the extent of cDNA degradation during the hybridizations, HAP chromatography, and other steps involved in preparation of subtractive cDNA, as observed also by others(3). A portion of the size-selected, PCR- amplified and -chased material was cloned into λgt10 as described in Materials and Methods.

We used three approaches for analyzing our subtractive cDNA library: 1) differential hybridization with labelled first strand 4N2.1 and A20.2J cDNA probes; 2) hybridization with a portion of the amplified subtractive cDNA insert; and 3) analysis of random clones.

Differential hybridization of 1290 plaques resulted in 10 plaques (0.8%) which gave equally strong positive signals with the first strand cDNA probes derived from either 4N2.1 or A20.2J RNA. These clones presumably derive from mRNAs which are present at high abundance in both cell lines but which were not removed completely by the subtraction. In addition,

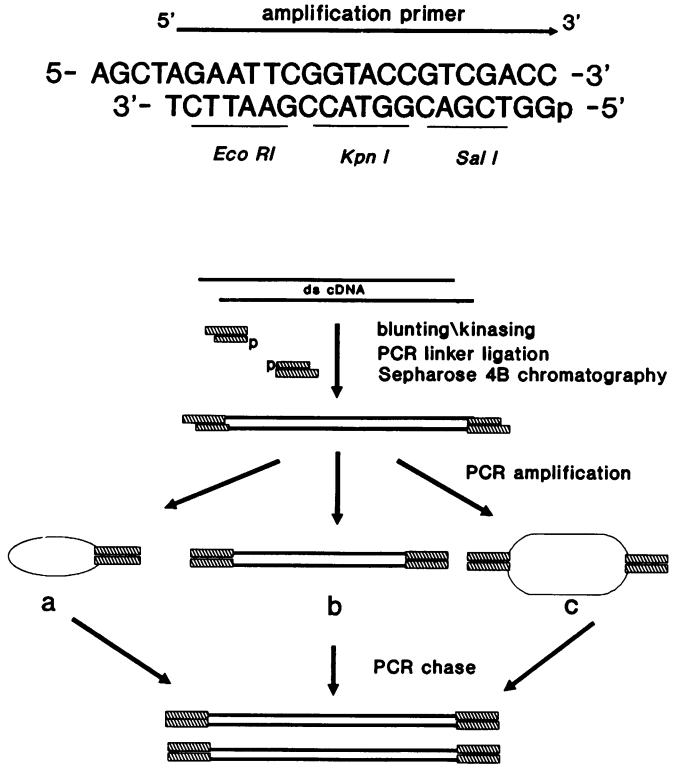

Figure 1. Schematic representation of amplification linker and PCR amplification of subtractive cDNA. Each strand of the amplification linker is shown as a rectangular box with hatched lines. Details of various procedures are included in the Materials and Methods.

183 plaques (14%) showed a significantly stronger positive signal with the 4N2.1 first strand cDNA probe than with the A20.2J first strand cDNA probe. These clones must be generated from highly abundant mRNAs that are present at higher levels in the 4N2.1 cell line than in the A20.2J cell line. All 183 plaques in this second category reacted strongly with a 4.3 kb XbaI/BamHI genomic fragment containing the mouse kappa light chain constant region(26). Inserts from several of these plaques identified a 0.8 kb mRNA in the parental 4N2.1 cells, and a 1.3 kb mRNA in 653FB and A20.2J cells (10, 11). These cDNA clones must have been derived from the kappa light chain constant region fragment mRNA, but were incompletely subtracted (see Discussion).

Hybridization of the 1290 plaques with a subtractive insert probe resulted in identification of 39 (3%) new plaques in addition to the plaques containing kappa light chain cDNA sequences. We analyzed 14 of these plaques by isolating insert and probing Northern blots to assess expression in the 4N2.1 parental plasmacytoma line, an unrelated plasmacytoma line called 653FB, and the A20.2J B lymphoma subtractive partner. Two clones were nonsubtractive, i.e. they identified similar amounts of mRNA in all three cell lines. Eleven clones were tumor specific, i.e. they recognized a specific mRNA in the 4N2.1 parental cell line but not in the other two cell lines. Ten of these tumor specific clones identify an approximately 1 kb mRNA. Only a single clone was qualitatively subtractive, identifying a 2.0 kb mRNA both in the 4N2.1 and 653FB plasmacytoma cell lines but not in the A20.2J subtractive partner. On a preliminary screen of a panel of B cells this latter clone identified the same 2.0 kb mRNA in the WEHI 231 immature B cell line and in the 70Z/3B pre-B lymphoma cell line (C. Timblin and M. Kuehl, unpublished).

TABLE II. Analysis of 115 Clones from 4N2.1 Plasmacytoma minus A20.2J B lymphoma Subtractive cDNA Library for differential RNA Expression.*

	Number (%)	
No Signal	26	(23)
Non-subtractive	20	(17)
Subtractive	69	(60)
Tumor Specific	14	
Quantitative	16	
Qualitative	39	

* An insert from each λgt10 clone was used to screen Northern blots (See Results).

Based on the first two screening procedures, it is apparent that plasmacytoma-specific mRNAs that are expressed in 4N2.1 and not in A20.2J cells must occur principally in the low abundance and rare classes. We next attempted to: 1)identify potential plasmacytoma-specific mRNAs in low abundance and rare classes; and 2)better define the characteristics of our 4N2.1 minus A20.2J subtractive cDNA library. To do this we screened 129 plaques (excluding plaques containing kappa light chain sequences) at random from the grid. We used the A pair of λgtl0 oligonucleotides (Materials and Methods) to generate an insert from each plaque. For 14 plaques, we were unable to identify an insert by analysis of the PCR reaction on a 1.5% agarose gel. But for 3 of the 14 plaques we could identify a fragment of 58 +/− a few bp on a nondenaturing 8% polyacrylamide gel, indicating that these three plaques contained phage lacking inserts. Repeated attempts to generate inserts from the other 11 phage using PCR reactions with either the A or B pairs of λgtl0 oligonucleotides failed to produce any kind of insert. Thus about 2% of the phage in our library are proven to lack inserts and about 9% fail to generate inserts by the PCR reaction.

The results for the remaining 115 phage plaques are summarized in Table II. In each case, we generated an insert by PCR reaction, and purified the DNA fragment on an agarose gel. Most of the inserts contained 300−500 bp. The labelled fragment was used to probe Northern blots derived from gels loaded with 10 μg per lane of 4N2.1 or 653FB plasmacytoma or A20.2J B lymphoma total cytoplasmic RNAs. For the 32 (28%) inserts which generated no signals on these blots, we then probed an additional Northern blot, and, in some cases, a Southern blot. The additional Northern blots were derived from gels loaded with 5 μg per lane of 4N2.1 or A20.2J poly(A)+ cytoplasmic RNA, and the Southern blots were derived from agarose gels loaded with 5 ug each of EcoRI and BamHI digested 4N2.1 genomic DNA per lane. Twenty-six (23%) inserts gave no signal on either set of Northern blots; of the 16 of these inserts used to probe Southern blots, 11 generated a signal and 5 gave no signal.

The remaining 89 (77%) inserts included 4 patterns of reactivity, i.e. non-subtractive, tumor specific, quantitatively subtractive and qualitatively subtractive, as shown in Fig. 2 and described below. Twenty (17%) inserts generated a non-subtractive signal, i.e. they detected the same mRNA in the parental 4N2.1 myeloma cells and in the A20.2J subtractive partner, with little or no significant difference in the levels of expression.

Sixty-nine (60%) inserts generated a subtractive signal. These could be arranged into three categories. Sixteen (14%) were quantitively subtractive, with expression levels approximately threefold or more in 4N2.1 compared to A20.2J cells. Fourteen (12%) inserts generated tumor specific signals, i.e. expression

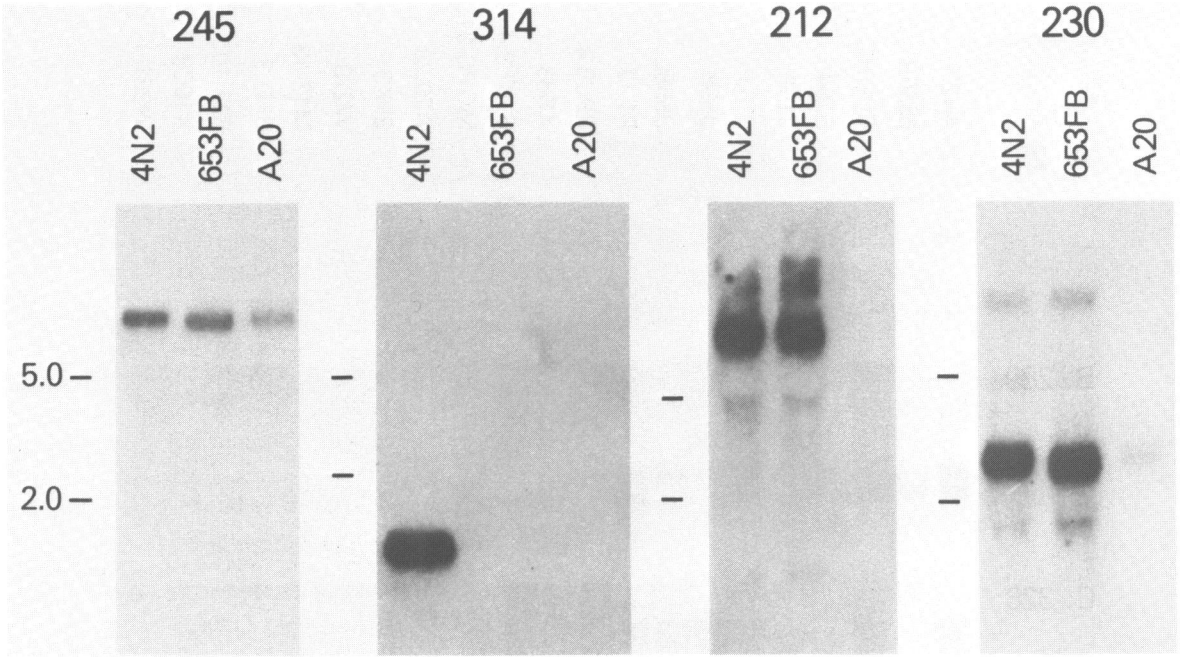

Figure 2. Examples of Northern blots for nonsubtractive, tumor specific, qualitatively subtractive and quantitatively subtractive clones. Ten micrograms of total cytoplasmic RNA from the 4N2.1 parental plasmacytoma cell line, the unrelated 653FB plasmacytoma cell line, and the A20.2J subtractive partner B lymphoma cell line were fractionated by electrophoresis. The resultant Northern blots were hybridized with random primer labelled DNA inserts from clones 245, 314, 212, and 230. The position of the 28S(5.0kb) and 18S(2.0kb) rRNAs are indicated by the horizontal marks. Clone 245 represents a nonsubtractive clone (4 hr exposure); 314, a tumor specific clone (4 hr exposure); 212, a qualitatively subtractive clone (14 hr exposure); and 230, a quantitatively subtractive clone (5 hr exposure).

of mRNA in 4N2.1 cells but not in 653FB plasmacytoma cells or in A20.2J cells. Nine of the fourteen detected a 1 kb mRNA (Fig. 2). These nine inserts cross-hybridized with each other and also with the tumor specific clones identified by probing with the subtractive insert (see above). Curiously, the sequence of one of these inserts (L. Brents and C. Timblin, unpublished) demonstrated identity to kappa-casein, a gene expressed in mammary tissues (27) but not in any of the 13 hematopoietic cell lines examined by us (data not shown). Thirty-nine (34%) inserts were qualitatively subtractive, i.e. detecting the same mRNA species in 4N2.1 and 653FB plasmacytoma cells but not detecting an mRNA in A20.2J cells.

Excluding the tumor specific clones, very few sibs (i.e. defined as cDNA clones which recognize the same mRNA) were identified in this analysis of 115 clones. There was only one example of a pair of sibs for the 16 quantitatively subtractive clones. For the 39 qualitatively subtractive clones there were five sets of sibs, i.e. three sets with a pair of sibs and two sets with three sibs. Thus qualitative subtractive cDNA clones recognizing 32 different mRNAs were obtained in this analysis of 115 random clones.

Twenty-nine of the unrelated qualitatively subtractive clones were screened against panels which included two additional plasmacytomas (CBOHC, S194), three additional B lymphomas (2PK3, M12.13, WEHI 231), and one pre-B lymphoma (70Z/3B) (data not shown). Three of these clones identified mRNA in at least one of the two additional plasmacytomas but none of the four B and pre-B lymphomas. Inserts from these three clones were hybridized to an expanded panel of plasmacytoma and B lymphoma cell lines (Fig. 3). Each clone detected mRNA in at least 10 of 11 independent plasmacytomas examined (we note that clone 251 detects mRNA in 315J, but not in 315P, a nonsecreting variant of 315J-ref. 31). In contrast mRNA was not detected in 8 independent B lymphoma cell lines (Fig. 3) or in 10 pre-B lymphoma cell lines (data not shown). Thus each of these three unrelated clones apparently provides a marker which distinguishes terminally differentiated plasmacytomas from less differentiated B or pre-B lymphoma cell lines.

DISCUSSION

In this manuscript we describe a simple method for preparing a renewable source of subtractive cDNA which can then be used as a hybridization probe or as insert which can be cloned into a variety of convenient vectors. This has been accomplished by using a double-stranded oligonucleotide which has a 5′-phosphate at its blunt end and a non-phosphorylated 5′ overhang at the other end (Fig.1), so that the blunt end can be ligated to any DNA molecule which has a blunt end containing a 5′-phosphate. When this oligonucleotide is ligated to each end of a piece of DNA, the DNA can be PCR amplified with a single appropriate oligonucleotide. Although the presence of the same oligonucleotide linker at each end of a piece of DNA provides for the possibility of forming self-annealed structures (Fig. 1), it appears that this does not prevent efficient PCR amplification.

As experience with PCR amplification has increased in recent years, it is apparent that not all pieces of DNA can be amplified with the same efficiency (28). The reasons for this are poorly understood, although it seems clear that smaller molecules (i.e. less than 1 kb) are more likely to amplify efficiently than larger molecules (29). The application of PCR technology to subtractive cDNA amplification seems favorable since the subtractive cDNA molecules tend to have a restricted size heterogeneity of approximately 300−500 bp. We have no direct evidence bearing on the question of differential amplification of heterogeneous

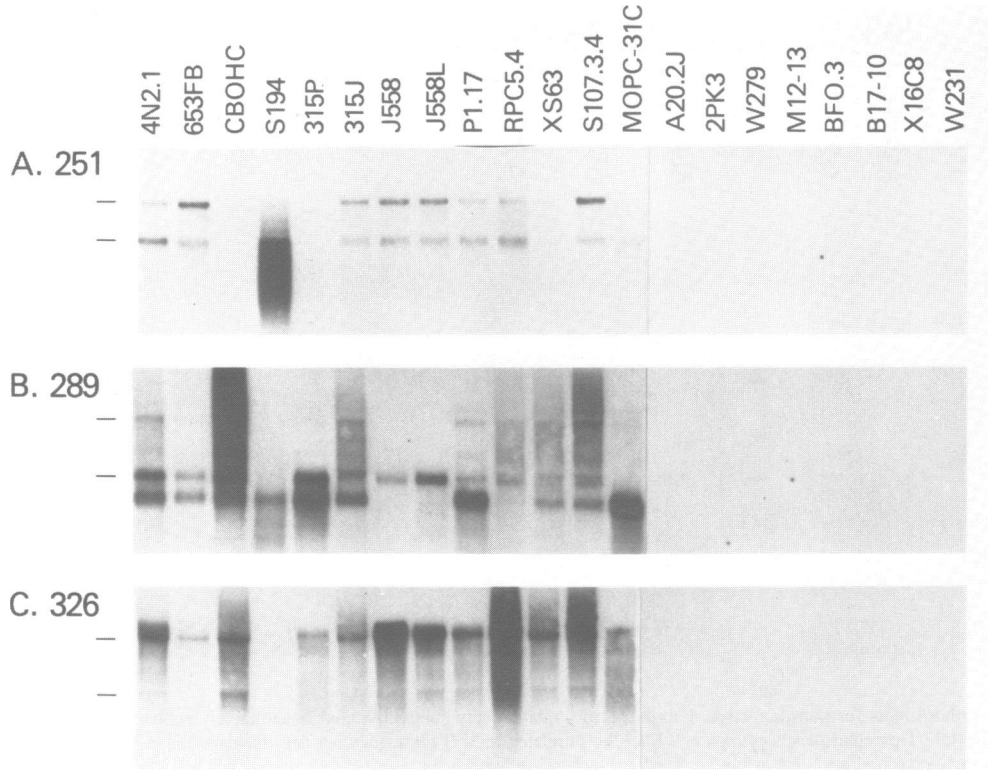

Figure 3. Expression of three qualitatively subtractive clones in plasmacytoma and B lymphoma cell lines. Northern blot panels of 13 plasmacytoma cell lines and 8 B lymphoma cell lines were prepared from gels loaded with 5μg of each poly(A+)RNA. The blots were hybridized with random primer labelled DNA inserts from three clones: A, clone 251 (67 hr exposure); B, clone 289 (96 hr exposure); C, clone 326 (16 hr exposure). Indicated above each lane is the cell line used: 4N2.1, parental plasmacytoma; 653FB, CBOHC, S194, 315P, 315J, J558, J558L, P1.17, RPC5.4, XS63, S107.3.4, and MOPC-31C are other plasmacytomas; A20.2J, the subtractive B lymphoma partner; 2PK3, WEHI 279, M12−13, BF0.3, B17−10, X16C8, and WEHI 231 are other B lymphoma cell lines. The J558 and J558L cell lines are related sublines derived from the J558 plasmacytoma tumor. Plasmacytoma cell line 315P is an IgA, low secreting variant of 315J (31). The positions of 28S and 18S rRNAs are indicated by the horizontal marks.

populations of subtractive cDNAs. However, it is encouraging that dT-primed and random-primed subtractive libraries (prepared by the method described here from parental Swiss 3T3 RNA and subtractive partner Balb 3T3 RNA) each generated qualitatively subtractive clones encoded by the same 20 moderately abundant mRNAs; these clones were identified by hybridization to the dT- or random-primed subtractive insert, respectively (J. Battey, unpublished).

The initial analysis of our 4N2.1 minus A20.2J subtractive cDNA library indicates that this approach has been technically successful. Since we used only an estimated 250 μg of 'pure' A20.2J mRNA for two subtractions compared to the 50 μg of 4N2.1 mRNA used as template for first strand cDNA synthesis, the incomplete subtraction of cDNA derived from the highly abundant mRNA for the kappa constant region fragment is not surprising, i.e. kappa light chain constant region mRNA sequences are represented at a twenty-fold lower level in A20.2J than in 4N2.1(10,12). Excluding the kappa clones and clones which had no insert detectable by PCR, 46% of the clones were qualitatively subtractive (including tumor specific in this category) and 14% of the clones were quantitatively subtractive. Only 17% of the clones were non-subtractive. The remaining 23% of the clones generated a PCR insert which gave no detectable signal with a Northern blot prepared from a gel loaded with 5 μg of poly(A)+ cytoplasmic RNA per lane. In this 'no signal' category, 11 of 16 clones examined did hybridize to a band on a genomic Southern blot. It is likely that most of these clones were generated from trace amounts of contaminant DNA present either in the large quantities of RNA used for hybridization or in the reagents used to prepare the subtractive cDNA. It is also possible that some of these clones are derived from intronic sequences present in nuclear RNA contaminating our cytoplasmic RNA preparations, or from mRNAs expressed at less than a copy per cell.

From the 1.75% calculated yield of first strand cDNA after two rounds of subtraction and one round of positive selection, we estimate that qualitatively subtractive sequences should be enriched about 60-fold relative to the unselected first strand cDNA. In support of this estimate, we have used one qualitatively subtractive clone (i.e. clone 289−Fig. 3) to screen both an unsubtracted plasmacytoma cDNA library and the subtracted cDNA library described here. The frequency of positive clones was one per 1000 in the subtractive library and 1 per 20,000 in the unsubtracted library, consistent with a 60-fold enrichment of this sequence in the subtractive library since the average insert was approximately three times as large in the unsubtracted library as in the subtractive library (C.Timblin and L. Brents, data not shown). Similar results were obtained with a number of qualitatively subtractive clones derived from the Swiss 3T3/Balb 3T3 library mentioned above (J. Battey, unpublished).

Despite the technical success in generating this 4N2.1 minus A20.2J cDNA library, our initial analysis provides only two preliminary insights regarding the difference in gene expression in a mouse plasmacytoma vs. a highly differentiated mouse B

cell lymphoma. First, there are no highly abundant, and few—if any—moderately abundant mRNAs which are expressed uniquely in the plasmacytoma cells. Second, of 29 different qualitatively subtractive clones analyzed, only 3 detect consensus differences between plasmacytoma and B cells, even though the clones in this group of 29 are more likely to react with an unrelated plasmacytoma cell line than an unrelated B lymphoma cell line (C.Timblin and M. Kuehl, unpublished).

Since most of the qualitatively subtractive clones in this library are in the low abundance or rare categories, it is worthwhile pointing out how much of the library we have been able to screen thus far. Assuming about 250,000 mRNAs with an average size of 2000 bp in a plasmacytoma cell line(30) and a 60 fold enrichment by subtraction (see above), the 'complexity' of the subtracted cDNA library would be roughly 4000 mRNAs. In analyzing 115 random clones (Table II), we estimate that we have screened approximately 3% of this 'complexity'. In other words, we could have missed clones which would detect mRNA species that are expressed at 20−30 copies per cell. We can make only a rough guess as to the number of different mRNAs which are expressed in 4N2.1 plasmacytoma cells and not in A20.2J B lymphoma cells. Assuming that the average mRNA encoding a qualitatively subtractive cDNA is present at 5−10 copies per cell, we estimate that the plasmacytoma cell contains $32/(0.03\times5-10)$, or approximately 100−200 different 'qualitatively subtractive mRNAs.'

By using our subtractive insert which already contains amplification linkers, we are in the process of developing a strategy which will enable us to re-subtract this insert against a panel of B cell and pre-B cell lymphomas. If we are able to achieve additional subtractions and positive selection steps starting with the present subtractive insert, it may be possible to decrease the 'complexity' of the secondarily subtracted library to several hundred clones;this should enhance our chances of identifying additional consensus plasmacytoma-specific mRNAs. In any case, the PCR strategy described here for preparation of a renewable source of subtractive cDNA should facilitate the general use of subtractive cDNA for assessing cell specific differences in gene expression.

ACKNOWLEDGEMENTS

We especially thank George Yancopoulos and Fred Alt for sharing protocols, results, and ideas regarding subtractive cDNA technology; and Michael Birrer for helpful discussions on PCR technology. We also wish to thank Leslie Brents for technical assistance, as well as Joan Linville and Gail Gray for assistance in the preparation of the manuscript.

REFERENCES

1. Sargent, T.D.(1987) Methods in Enzymology, 152, 423−432.
2. Alt, F.W., Kellems, R.E., Bertino, J.R. and Schimke, R.T. (1978) J. Biol. Chem., 263, 1357−1370.
3. Sargent, T.D. and Dawid, I.B. (1983) Science 222, 135−139.
4. Hedrick, S.M., Cohen, D.I., Nielsen, E.A. and Davis, M.M. (1984) Nature 308, 149−153.
5. Sakaguchi, N., Berger, C.N. and Melchers, F. (1986) The EMBO Journal 5, 2139−2147.
6. Travis, B.H. and Sutcliffe, J.G. (1988) Proc. Nat. Acad. Sci., 85, 1696−1700
7. Duguid, J.R., Rohwer, R.G. and Seed, B. (1988) Proc. Nat. Acad. Sci. 85, 57318−5742. (1981), 369−378.
8. Mather, E.L., Alt, F.W., Bothwell, A.L.M, Baltimore, S. and Koshland, M.E. (1981) Cell 13, 369−378.
9. Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Cell 51, 987−1000.
10. Choi, E., Kuehl, W.M. and Wall, R. (1980) Nature 286, 776−779.
11. Kearney, J.F., Radbruch, A., Liesegang, B. and Rajewsky, K. (1979) J.Immunol. 123, 1548−1550.
12. Word, C.J. and Kuehl, W.M. (1981) Molec. Immunol. 18, 311−322.
13. Falvoro, J., Freisman, R., and Kamen, R. (1980) Methods Enzymol. 65, 718−749.
14. Chirgwin, J.M. , Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochem. 18, 5294−5299.
15. Brawerman, G. (1973) Methods in Cell Biology 7, 2−22.
16. Aviv, H. and Leder, P. (1972) Proc. Nat. Acad. Sci. 69, 1408−1412.
17. Lehrach, H., Diamond, D., Wozney, J.M. and Boedtker, H. (1977) Biochem. 16, 4743−4751.
18. Bender, T.P. and Kuehl, W.M. (1987) J. Immunol. 139, 3822−3827.
19. Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic Methods in Molecular Biology. Elsevier, New York.
20. Britten, R.J., Graham, D.E. and Neufeld, B.R. (1974) Methods Enzymol. 29, 363−418.
21. Matter, A., Lisowska-Bernstein, B., Ryser, J.E., Lamelin, J-P. and Vassalli, P. (1972) J. Exp. Med. 136, 1008−1030.
22. Potter, M. (1987) Adv. Viral Oncol. 7, 99−122.
23. Boothby, M., Gravallese, E., Liou , H-C. and Glimcher, L.H. (1988) Science 242, 1559−1562.
24. Cory, S. (1986) Adv. Ca. Res. 47, 189−234.
25. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
26. Seidman, J.G. and Leder, P. (1978) Nature 276, 790−795.
27. Thompson, M.D., Dave, J.R. and Nakhasi, H.L. (1985) DNA 4, 263−271.
28. Erlich, H.A. (Editor) (1989) PCR Technology. Stockton Press, N.Y.
29. Jeffreys, A.J., Wilson, V., Neumann, R. and Keyte, J. (1988) Nucleic Acids Res. 16, 10953−10971.
30. Perry, R.R. and Kelley, D.E. (1979) Cell 18, 1333−1339.
31. Gebel, H.M., Autry, J.R., Rohrer, J.W. and Lynch, R.G. (1979) J. Natl. Cancer Inst. 62, 201−211.