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The isolation of cloned cDNA sequences which are differentially expressed in human lymphocytes and fibroblasts

Julian Crampton, Stephen Humphries, Derek Woods and Robert Williamson

Biochemistry Department, St. Mary's Hospital Medical School, University of London, London, W2 1PG, UK

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ABSTRACT

Poly(A)$^+$ RNA populations derived from normal lymphocytes and fibroblasts have been compared by hybridising each RNA to cDNA derived from the other RNA population. This indicated that approximately 75% of the sequences were common to both, and that these were present at different concentrations in the two cell types. The two RNA populations were further compared by hybridising them to a cDNA recombinant library derived from lymphocyte poly(A)$^+$ RNA. This allowedthe identification of clones containing sequences which are abundant in lymphocyte poly(A)$^+$ RNA but absent or rare in fibroblast poly(A)$^+$ RNA. A direct estimation of the abundance of five of these sequences in lymphocyte cDNA demonstrated that clones can be detected by such a procedure if they represent 0.2% or greater of the original cDNA population.

INTRODUCTION

It has been known for some time that the poly(A)$^+$ RNA populations of different cell types differ in the sequences which are present and their relative abundances (1, 2). The availability of cloned libraries of expressed gene sequences has stimulated detailed molecular analysis both of individual RNAs and of RNA populations. Screening of such libraries with a complex RNA probe (such as that derived from total cellular poly(A)$^+$ RNA) is limited by the inability to detect clones containing sequences present in low copy number in the probe. Thus only clones containing sequences which code for the middle and highly abundant RNAs can be identified using the Grunstein-Hogness procedure (3).

Gergen et al. (4) have reported a model clone search using different mixtures of probes to analyse the limit of the Grunstein-Hogness screening method. They calculate a theoretical limit of detection where 0.1% of the probe is complementary but find a practical limit of 0.5% using reasonable probe concentrations, hybridisation and autoradiographic exposure times.

A useful application of cDNA libraries will be to compare RNA populations derived from a number of cell types. Such comparisons will

allow the isolation of sequences that are specific to a particular cell type or those which are expressed in all cells. It is possible to screen clones of one cDNA library with RNA probes derived from a variety of cell types. Such analyses have proved successful in the isolation of differentially expressed sequences, e.g. from Silkmoth and Xenopus cDNA libraries (5, 6).

This paper describes a direct estimation of the abundance of sequences hybridising to clones selected by such a comparative screening, using the differences between human lymphocyte and fibroblast poly(A)$^+$ RNA as a model system. A lymphocyte cDNA library was hybridised at high density with RNA probes isolated from fibroblasts and lymphocytes. Some clones hybridised to sequences present in lymphocyte RNA but could not be detected with the fibroblast probe. These were picked, purified and the abundance of their complementary sequences in lymphocyte cDNA was determined by hybridising this cDNA to recombinant DNA immobilised on filters. This gave an estimate of the sensitivity of the comparative screening procedures.

MATERIALS AND METHODS

Preparation of normal human lymphocyte and fibroblast poly(A)$^+$ RNA

Normal human lymphocytes were prepared from buffy coat residues as described by Harris et al. (7). Normal human fibroblasts were obtained from a skin biopsy grown in culture in BHK-GMEM supplemented with 10% foetal calf serum. Total RNA was prepared from both cell types by a modification of the method of Strokman et al. (8) as described by Woods et al. (9). Poly(A)$^+$ RNA was purified using oligo (dT) cellulose chromatography (10).

Labelling of nucleic acids

RNA was labelled in vitro with polynucleotide kinase (P.L. Biochemicals). The RNA was hydrolysed to an average length of 100-200 bases using 0.1N NaOH at 0° for 15 mins. before labelling with γ-^{32}P-ATP (11). Specific activity of the RNA was about 10^7 cpm/μg.

^{32}P cDNA was prepared as described by Woods et al. (9) to a specific activity of 5 x 10^6 cpm/μg using α-^{32}P-dCTP and ^{3}H cDNA was prepared for RNA:cDNA hybridisation using ^{3}H-dCTP to a specific activity of 10^7 cpm/μg also as described by Woods et al. (9).

RNA:cDNA hybridisation

RNA:cDNA hybridisations and their analysis by S1 nuclease digestion was performed as described by Getz et al. (12).

Manipulation of the recombinant cDNA library

All manipulations of recombinants were carried out under CII containment conditions in accordance with the G.M.A.G. guidelines for recombinant DNA research.

The cloned library derived from normal human lymphocyte cDNA was that described by Woods et al. (9). Approximately 400 recombinants were filtered directly onto 9cm nitrocellulose filters. These masters were grown at 37° on 10µg/ml tetracycline until the colonies were well established. Four replicas were made of each master on nitrocellulose filters. Locating marks were made within each filter for future orientation. The replicas were grown until the colonies were approximately 1mm in diameter, the filters were then transferred to chloramphenicol plates and incubated overnight to amplify the plasmids present in the clones. Masters were kept at 4° until required.

Colonies on nitrocellulose filter discs (either random spreads or ordered arrays) were lysed and their DNA immobilised to the nitrocellulose essentially as described by Grunstein and Hogness (3), except that the filters were not treated with proteinase K or washed with chloroform.

Hybridisation of ^{32}P poly(A)$^+$ RNA to colony DNA on filters

Filters to be hybridised were prewashed in 50% formamide, 0.1% SDS, 5 x SSC, 50µg/ml carrier DNA, 10µg/ml poly(A) and 1 x Denhardt's solution (13) at 42°. Hybridisation with ^{32}P RNA was carried out in the same prewash buffer (1ml per 9cm filter disc) in heat sealed plastic bags at 42° for 16 hours. After hybridisation the filters were washed four times in 2 x SSC, 0.1% SDS at 65° and twice in 0.1 x SSC, 0.1% SDS at 65°. The filters were air dried and exposed to preflashed Fuji X-ray film with an intensifying screen at -70°.

Plasmid DNA preparation

Plasmid DNA was prepared from 1L cultures of selected clones by the detergent lysate method of Clewell and Helinski (14). The DNA was purified by centrifugation in ethidium bromide/caesium chloride density gradients (15).

Hybridisation of ^{32}P cDNA to immobilised plasmid DNA

Denatured plasmid DNA was loaded onto 25mm nitrocellulose discs as described by Birnsteil et al. (16). In vivo ^{3}H-labelled pAT153 plasmid DNA was included during loading of the filters as an internal standard to allow an estimate of the amount of input plasmid DNA binding to each filter. This was routinely 75%-80%. Filters were washed prior to hybridisation in 3 x SSC, 0.1% SDS, 50µg/ml carrier DNA, 10µg/ml poly(A)$^+$ 1 x Denhardt's

solution (13) at 65°. Filters were hybridised to each ^{32}P cDNA probe in a heat sealed plastic bag in 5ml of prewash buffer for 16 hours at 65°. A known amount of mouse globin ^{32}P cDNA was added to each hybridisation bag and a set of filters loaded with DNA from a mouse globin cDNA plasmid was included as a kinetic standard.

After hybridisation the filters were washed exhaustively in 2 x SSC, 0.1% SDS at 65° by a batch procedure. They were then immersed in 20ml of 70mM NaAcetate, 2.8mM Zn_2SO_4, 140mM NaCl pH 4.5. 40 units of S1 nuclease wereadded and the whole incubated at 37° for one hour. The filters were then washed twice more in 2 x SSC, 0.1% SDS at 65°, dried in an oven and counted in a toluene based scintillant.

RESULTS AND DISCUSSION

Kinetics of hybridisation of poly(A)+ RNA to homologous and non-homologous cDNA

Fibroblast and lymphocyte poly(A)$^+$ RNA was initially analysed by kinetic hybridisation to estimate the degree of homology between the two RNA populations. A 50 fold excess of poly(A)$^+$ RNA was hybridised with its complementary DNA and the extent of hybridisation assayed by resistance to S1 nuclease. The kinetics of hybridisation are shown in Fig. 1A and B. A computer analysis of this data, using mouse globin RNA:cDNA as a kinetic standard, was carried out by Dr. Bryan Young (Beatson Institute, Glasgow) and the results are shown in Table 1. It indicates that there are 6-8000 different poly(A)$^+$ RNAs in each cell type. The degree of homology between the two populations of poly(A)$^+$ RNA was determined by cross hybridisation between these RNAs and their cDNAs (Fig. 1C and D). In both cases only 63-65% of the cDNA hybridised to the non-homologous RNA at the highest Rot values tested in comparison with 80-82% in the homologous reaction. This indicated that about 25% by weight of the sequences present in each cell type were absent or extremely rare in the RNA population of the other cell type. Furthermore, it can be seen that each RNA drives the non-homologous cDNA into hybrids more slowly (by 1 log Rot unit) than its homologous cDNA indicating that some of the sequences common to both are present at different abundances.

The above data suggest that screening a lymphocyte-derived cDNA library with the two poly(A)$^+$ RNA probes would reveal some clones hybridising with a lymphocyte probe which do not hybridise with a fibroblast probe. Clones thus selected should code for sequences abundant in lymphocytes but rare or absent in fibroblasts. (The reciprocal experiment with a fibroblast-derived cDNA

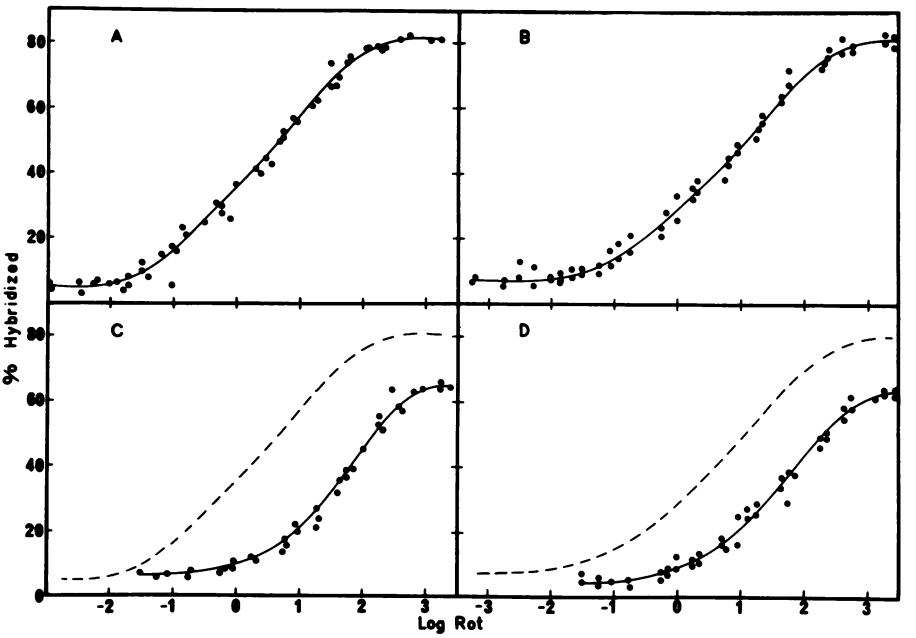

Figure 1 Homologous and non-homologous hybridisation of lymphocyte and fibroblast poly(A)$^+$ RNA to ^{3}H-cDNA.
(A) Lymphocyte poly(A)$^+$ RNA to lymphocyte cDNA. (B) Fibroblast poly(A)$^+$ RNA to fibroblast cDNA. (C) Lymphocyte poly(A)$^+$ RNA to fibroblast cDNA. (D) Fibroblast poly(A)$^+$ RNA to lymphocyte cDNA. The dashed lines in C and D show the curves from A and B respectively.

library would select for clones complementary to abundant fibroblast RNAs which are rare in lymphocytes.)

Screening of a lymphocyte cDNA library with kinase-labelled lymphocyte and fibroblast poly(A)$^+$ RNAs

Initial high density screening of the lymphocyte cDNA library was carried out as described in the Methods section. Using replicas, each master filter, containing about 400 recombinants, was screened in duplicate with kinase-labelled lymphocyte and fibroblast poly(A)$^+$ RNA. Fig. 2A and B shows the result of a typical high density screening with the two probes. Clearly a number of colonies where hybridisation has occurred with the lymphocyte probe show a reduced or absent signal with the fibroblast probe (the dark area in A resolves into individual colonies on shorter exposure of the autoradiograph). 120 clones gave strong hybridisation signals with the ^{32}P labelled

TABLE 1

Source of RNA and component (a)	% of cDNA hybridised (b)	$Rot_{\frac{1}{2}}$ (c)	$Rot_{\frac{1}{2}}$ (corrected) (d)	Number of 1000 bp sequences (e)	Relative copy number
Lymphocyte					
1	54	22	.119	35	260
2	46	56	26	7800	1
Fibroblast					
1	43	22	.09	27	150
2	57	31	18	5400	1

a) The Rot curves in Fig. 1., A and B were divided into two components of 2 log units/component.
b) Percent of cDNA in each component after normalising the curves in Fig. 1.
c) Rate at which component is 50% hybridised.
d) $Rot_{\frac{1}{2}}$ corrected for the percentage of the cDNA in each component.
e) Calculated using mouse globin mRNA:cDNA hybridisation as a standard and assuming the average size of a mRNA to be 1000 bases.

lymphocyte RNA probe, of which 92 gave a clearly visible positive signal with fibroblast RNA of the same specific activity. These figures represent 30% and 23% of the plated recombinants respectively. The clones of interest were marked on the autoradiograph and this was used to pick the region of the master into microtitre wells for further analysis by the Grunstein-Hogness procedure. This was necessary because, at high clone density, it is not always possible to select a single hybridising clone unambiguously.

Figure 3 shows a typical Grunstein-Hogness screening of some of the selected clones with a lymphocyte RNA probe. Clones were restreaked on plates containing tetracycline and individual colonies rescreened as above to ensure their purity. The five clones indicated in Fig. 3 were grown in bulk and plasmid DNA was prepared for further investigation. These clones were chosen because they represented a range of hybridisation signal intensity when screened with lymphocyte RNA and all gave no positive signal when hybridised with fibroblast RNA. Clone pJL 17 gave the most intense signal and pJL 16 was barely detectable. Recombinant pJL 25 gave no

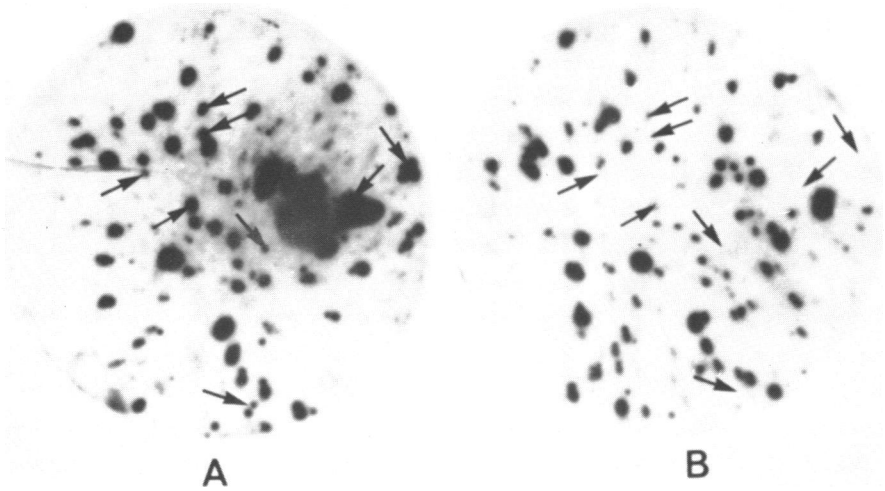

Figure 2 Random spreads of lymphocyte cDNA recombinant clones hybridised with ^{32}P lymphocyte poly(A)$^+$ RNA (A) and ^{32}P fibroblast poly(A)$^+$ RNA (B). The arrows indicate some of the clones which hybridise to lymphocyte RNA but not to fibroblast RNA. 0.5ng of ^{32}P-RNA was hybridised to each filter in 0.5ml for 16 hours at 42 °. Exposure was for 7 days.

positive signal with either lymphocyte or fibroblast probe and was used as a control in the hybridisation analysis described below. The clones described here did not hybridise to labelled rRNA.

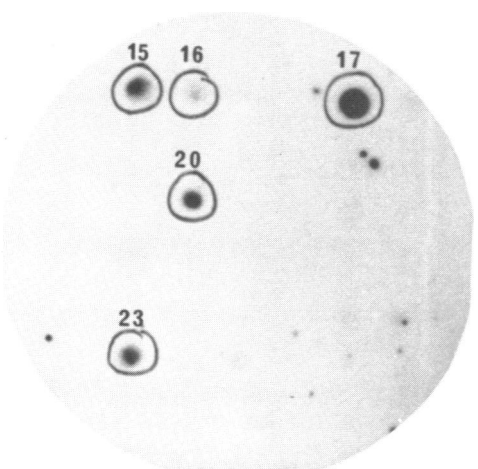

Figure 3 Ordered array of picked clones hybridised to ^{32}P lymphocyte poly(A)$^+$ RNA. The numbers are those referred to in the text. Hybridisation was as in Figure 2. Exposure was for 3 days.

Analysis of the purified clones

The purified recombinant plasmid DNA was linearised with EcoRI to give an estimate of the insert size. In no case was an EcoRI site detected within the insert, and this was confirmed by carrying out a Southern transfer (17) of the selected plasmids digested with EcoRI. The transferred DNA was hybridised to ^{32}P kinase-labelled lymphocyte and fibroblast poly(A)$^+$RNA. Figure 4 shows the pattern of hybridisation obtained with a lymphocyte poly(A)$^+$ RNA probe. For each plasmid the same amount of EcoRI-digested DNA was loaded. Clearly, hybridisation to the transferred DNA reflects differences in the hybridisation signals similar to those observed in the original Grunstein-Hogness screening. (No hybridisation was detected with fibroblast RNA [not shown].) The sizes of the inserts were calculated by comparison with markers of known size provided by HindIII/EcoRI double digest of DNA. pJL 17, with an insert size of 1.05 kb, clearly has a more intense signal than pJL 20 or 23, with inserts of 1.5 kb and 1.9 kb respectively. This clearly indicates that the most intense hybridisation signal was not due

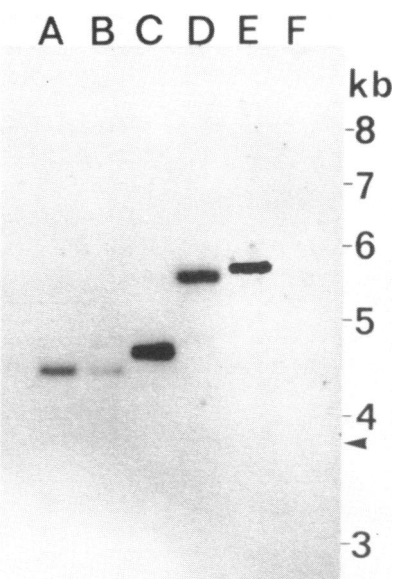

Figure 4 Southern transfer of EcoRI digested pJL clones hybridised to ^{32}P lymphocyte poly(A)$^+$ RNA. (A) pJL 15, (B) pJL 16, (C) pJL 17, (D) pJL 20, (E) pJL 23, (F) pJL 25. The arrow indicates the position of linear pAT153 DNA. 1 µg of ^{32}P RNA in 10ml was hybridised to the filter in a bag at 42° for 16 hours. Exposure was for 7 days.

to the presence of the largest insert.

Direct estimates of the abundance of the cloned sequences in the total lymphocyte poly(A)$^+$ RNA were made. These estimates would indicate the sensitivity of the comparative screening method and may prove useful in deciding whether such a screening procedure would be suitable for other situations. There are a number of methods available for estimating the abundance of a particular cloned sequence in poly(A)$^+$ RNA or in its cDNA. We used a direct hybridisation method originally described by Gillespie and Spiegelman (18) and modified by Birnsteil et al. (16).

Increasing amounts of plasmid DNA were denatured and immobilised to individual nitrocellulose filter discs. These were hybridised to total lymphocyte and fibroblast ^{32}P labelled cDNAs. Background hybridisation was estimated by including a set of filters loaded with pAT153 DNA. Mouse globin cDNA was mixed with each probe so that 1% or 0.1% of the total sequence complexity was globin. Filters loaded with a mouse globin cDNA plasmid were included in the hybridisation reactions. A comparison of the levels of hybridisation to the selected clones with this globin kinetic standard gave a figure for the amount of the total cDNA complementary to each

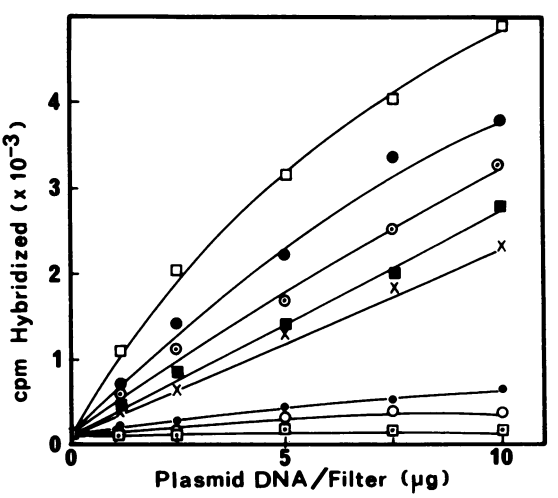

Figure 5 Hybridisation of ^{32}P lymphocyte cDNA to plasmid DNA immobilised on nitrocellulose filter discs. The immobilised DNAs are (□) pJL 17, (●) pJL 23, (⊙) globin hybridised with 1% of the probe being globin cDNA, (■) pJL 20, (X) pJL 15, (•) pJL 16, (O) globin with 0.1% of the probe being globin cDNA, (⊡) pJL 25 and pAT153.

cloned sequence.

The data obtained from this experiment are shown in Fig. 5. By comparing the initial gradients of each curve with that obtained using the globin standards representing 1% and 0.1% of the probe, the percentage of cDNA complementary to each plasmid was calculated; the results are given in Table 2. The limit of detection of the method with the specific activity and amount of cDNA used was 0.01% of the input cDNA. No detectable hybridisation occurred using the fibroblast cDNA probe ($\leq$0.01%).

pJL 17 appears to contain a sequence which represents a very abundant sequence in lymphocyte cDNA (amounting to 1.2% of the total). The nature of this sequence is under further investigation.

pJL 16, which gave the faintest detectable hybridisation signal in the

TABLE 2

Immobilised Plasmid DNA	Initial Gradient (a)	Size of Insert (Kb)	% of cDNA hybridised (c)
pAT153	0	0	-
Globin (b)	358	0.6	1.0%
Globin (b)	42	0.6	0.1%
pJL15	218	0.62	0.58%
pJL16	78	0.7	0.18%
pJL17	758	1.05	1.2%
pJL20	258	1.5	0.29%
pJL23	478	1.9	0.42%
pJL25	0.8	0.9	0.01%

a) The initial gradient of each curve was calculated and this figure is expressed in terms of cpm hybridised per µg plasmid on the filters.
b) Different percentages of globin cDNA were added to each bag in separate experiments.
c) This figure is calculated from the initial gradient of each curve by comparison with the mouse globin kinetic standard and corrected for the insert size in each case.

Grunstein-Hogness screening, accounted for approximately 0.2% of the lymphocyte cDNA. Thus, the Grunstein-Hogness screening is less sensitive than the quantitative analysis outlined above. The main limitations to the sensitivity of the colony hybridisation procedure at present are the specific activity of the probe and the limited hybridisation times possible with an RNA probe. Increasing the specific activity of the probe should allow the detection of clones complementary to as little as 0.05% of the RNA. Clearly, this will increase the proportion of the total sequences in a complex population which may be analysed in this way.

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