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Differential expression of selected genes in human leukemia leukocytes

(cDNA cloning/tissue-specific gene expression/nucleic acid hybridization)

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ABSTRACT **cDNA clones complementary to mRNA of cells from patients having chronic lymphocytic leukemia (CLL) were used to examine quantitative changes in the mRNA levels of specific genes in human leukemia leukocytes. Fourteen (of 400) CLL-positive clones that did not hybridize with placental mRNA were studied. Three of the 14 clones were highly represented in mRNA from CEM, a T-cell line. One clone was highly represented in mRNA from CLL and two were highly represented in mRNA from patients with chronic myelocytic leukemia. Ten clones were not significantly represented in normal leukocytes and spleen mRNA. We have identified several genes that are differentially expressed in human leukemia leukocytes.**

One view of the cause of cancer is that it results from an impairment of the cell differentiation process. That process may involve the transcription of genes not previously transcribed. More likely, it involves changes in the abundance of specific mRNAs in the cell. Thus, regulation involves modulation of the transcription and translation efficiencies of specific messengers. This argument predicts that the complexity of the mRNA population in most types of cells will be similar but the relative abundance of these messengers could vary widely. Messengers coding for enzymes found in high concentration in the cell (for example, glycolytic pathway enzymes) might fall into the high-abundance category, whereas rare proteins or genes regulated primarily by positive control would be transcribed at a low frequency.

Using various nucleic acid hybridization techniques, many investigators have found that changing patterns of RNA transcription occur during differentiation of eukaryotic cells and tissues (1–8). However, quantitative changes in mRNA levels accompanying neoplastic transformation have been difficult to measure (9).

By using recombinant DNA technology to prepare a cloned library of expressed gene sequences, discrete RNA species present in differential amounts in normal and malignant cells can now be examined. The relative abundance of a cloned sequence of cellular RNA can be estimated by nucleic acid hybridization.

Unique structural, biochemical, or biological features by which chronic lymphocytic leukemia (CLL) lymphocytes can be differentiated from normal lymphocytes have not been identified previously (10). mRNA from malignant B lymphocytes from a patient with CLL was used as material for a cDNA library. This library provides a resource for studying differential gene expression in hematopoietic cells. Several cDNA clones were identified that were differentially represented in the poly(A)$^+$RNA of various leukemia leukocytes.

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MATERIALS AND METHODS

Isolation of Poly(A)$^+$RNA from Normal Leukocytes, Leukemia Leukocytes, and Placenta. Normal and leukemia leukocytes obtained from peripheral blood were washed several times with Tris/NH_4Cl buffer (17 mM Tris·HCl/144 mM NH_4Cl, pH 7.2) and stored at −70°C.

CCRF-CEM cells were grown in continuous suspension culture in RPMI 1640 medium/10–20% fetal calf serum. This cell line was derived from a child with acute lymphocytic leukemia (ALL) and the cells have been characterized as thymus derived (11).

Total RNA from these cells or tissues was isolated by the method of Frazier *et al.* (12). Poly(A)$^+$RNA was isolated from total RNA by affinity chromatography on oligo(dT)-cellulose as described (13).

Preparation of cDNA Clones. Highly purified RNA-dependent DNA nucleotidyltransferase (reverse transcriptase) from avian myeloblastosis virus was kindly provided by J. W. Beard (Life Sciences, St. Petersburg, FL). cDNA was synthesized as described by Tashima *et al.* (14) and used as template for double-stranded cDNA synthesis with *Escherichia coli* DNA polymerase 1 (Boehringer Mannheim) as described by Efstratiadis *et al.* (15). The double-stranded DNA was digested with nuclease S1 (15) and deoxycytidine homopolymer tails were added by using terminal deoxynucleotidyltransferase (Bethesda Research Laboratories) as described by Roychoudhury *et al.* (16). Average tail length was estimated by incorporation of [^{3}H]dCTP (20 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) to be 9–14 residues. Deoxyguanosine homopolymers of 9–14 residues were added to the 3′ ends of *Pst* I-digested pBR322 (16).

A mixture of deoxycytidine-tailed double-stranded cDNA and deoxyguanosine-tailed linearized pBR322 in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.13 M NaCl was allowed to anneal for 4–6 hr at 65°C and cooled overnight (average rate, 2°C/hr). The annealed product was used to transform *E. coli* strain RR1 as described by Dagert and Ehrlich (17).

Colony Hybridization. Colonies grown on nitrocellulose filters were processed for hybridization essentially as described by Grunstein and Hogness (18). The filters were hybridized for 12 hr to [^{32}P]cDNA prepared from CLL, lymphoma, and CEM cells and placenta.

Dot Hybridization of ^{32}P-Labeled RNA to Recombinant Plasmids. RNA was partially hydrolyzed with 0.1 M NaOH for 15 min at 0°C and then neutralized with HCl. ^{32}P-Labeling of the free 5′-OH termini with T4 polynucleotide kinase was carried out according to Efstratiadis *et al.* (19).

Dot hybridization was carried out as described by Thomas

Abbreviations: CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia.

(20) with some modifications. Samples (1, 0.1, and 0.01 μg) of plasmid DNA were spotted directly onto dry nitrocellulose paper that had been soaked in H_2O, equilibrated with 3 M NaCl/0.30 M Na citrate and dried. The nitrocellulose papers were baked for 2 hr at 80°C. The prehybridization buffer was 50% (vol/vol) formamide/0.75 M NaCl/0.075 M Na citrate/Denhardt's solution. The DNA blots were prehybridized for 10–20 hr at 42°C. The hybridization buffer was 50% (vol/vol) formamide/0.75 M NaCl/0.075 M Na citrate/Denhardt's solution/0.1% $NaDodSO_4$ containing poly(A) at 20 μg/ml, tRNA at 100 μg/ml, and ^{32}P-labeled poly(A)$^+$RNA at 1×10^6 cpm/ml. The DNA blots were hybridized for 15–20 hr at 42°C and washed with four changes of 0.30 M NaCl/0.03 M Na citrate/0.2% $NaDodSO_4$ for 5 min each at room temperature, for 1 hr with three changes of the same buffer at 65°C, and then for 5 min with one change of 15 mM NaCl/1.5 mM Na citrate at room temperature. The filters were exposed to x-ray film at −70°C using a Kodak intensifying screen (Cronex Hi-plus).

RESULTS

Selection and analysis of clones containing copies of cDNA transcripts of poly(A)$^+$RNA was accomplished as shown below.

1. Preparation of cDNA library from total poly(A)$^+$RNA of CLL cells,
2. Screening of library with [^{32}P]cDNA from CLL, CEM (cell line derived from ALL), lymphoma, and placental cells,
3. Selection of 14 clones that react positively with [^{32}P]cDNA from CLL, CEM, and lymphoma cells but do not react with [^{32}P]cDNA from placental cells,
4. Purification of clones,
5. Determination of size of cDNA insert in each clone,
6. Dot hybridization of selected clones with [^{32}P]mRNA from CLL, CEM, lymphoma, chronic myelogenous leukemia (CML), B-cell lymphoma, normal leukocyte, and placental cells.

First, double-stranded cDNA was synthesized from poly(A)$^+$RNA from patients with CLL by using reverse transcriptase and DNA polymerase. Nuclease S1 was used to destroy the covalent linkage between the two strands. At this stage, the size of the molecules ranged from 100 to 2,000 base pairs as determined by agarose gel electrophoresis. The molecules were tailed with poly(dC) by using terminal deoxynucleotidyltransferase. Poly(dG) was added to the ends generated by a single *Pst* I cut in pBR322. The plasmid pBR322 normally confers ampicillin and tetracycline resistance to its host cell; however, insertion here was such that recombinant clones could be recognized as tetracycline resistant, ampicillin sensitive.

After transformation of *E. coli* K-12 strain RRI by 0.8 μg of recombinant plasmid DNA, approximately 12,000 tetracycline-resistant colonies were obtained. We picked 2,375 clones from this library and found that 60% of these clones reacted positively with [^{32}P]cDNA probes prepared from the homologous poly(A)$^+$RNA used in construction of the library. Three classes of clones were selected according to the intensity of the autoradiographic signal: high-, middle-, and low-intensity classes comprising 7%, 32%, and 21% of the clones, respectively. Clones containing cDNA transcripts of rare mRNAs and very short cDNA inserts would not be expected to react in the colony hybridization experiments.

Four hundred and sixty-two positive clones from the CLL cDNA library were used in the selection of specific genes that were represented in leukocyte RNA but not in placental RNA. [^{32}P]cDNA probes were prepared from poly(A)$^+$RNA of human placenta, CEM cells, and cells from patients with CLL and lym-

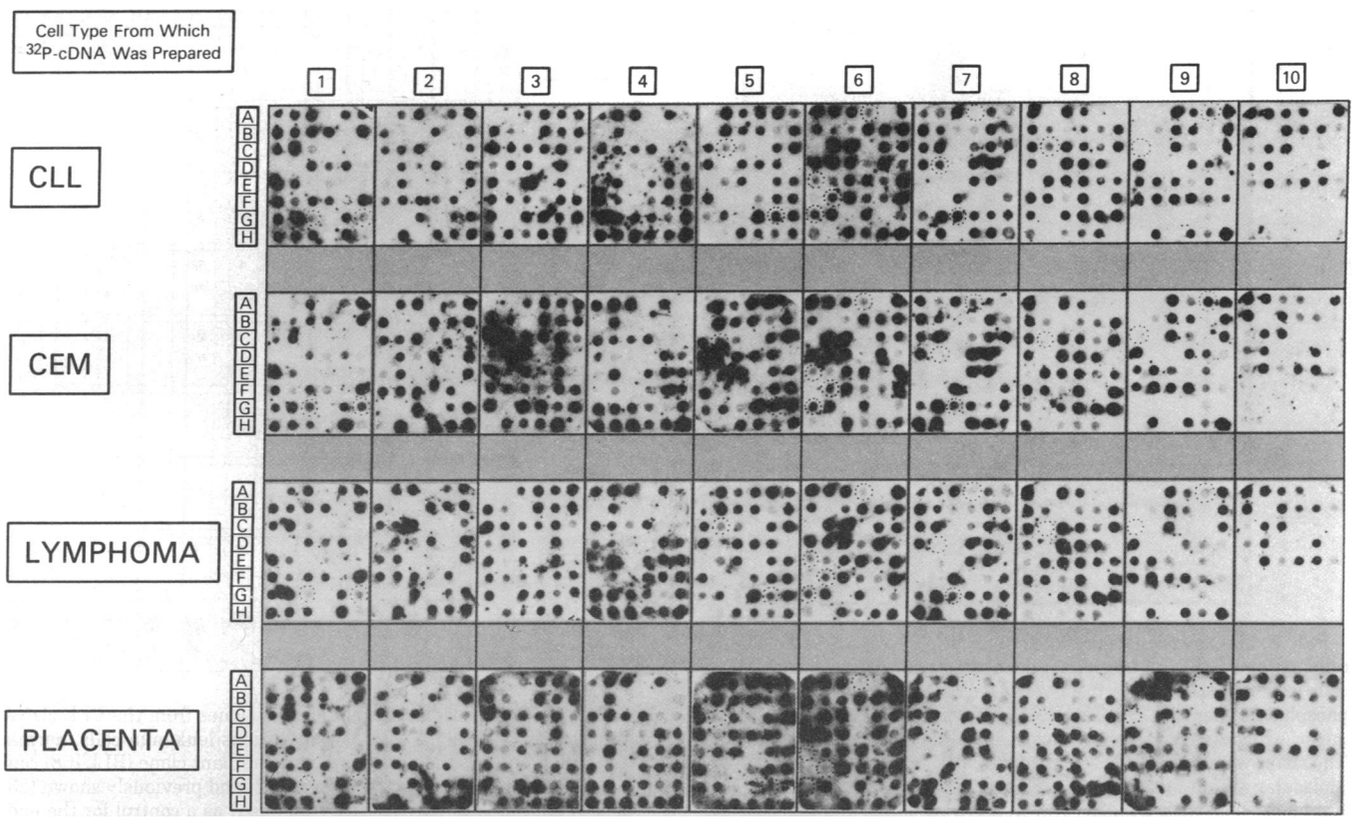

FIG. 1. Screening of the CLL cDNA library with [^{32}P]cDNA transcripts of poly(A)$^+$RNA prepared from CLL, lymphocyte line CEM (T cell), and leukemia lymphoma cells and from human placenta. Four hundred CLL cDNA clones were selected randomly for testing from 1,425 CLL-positive clones. Dotted circles identify selected clones that reacted positively with leukocyte cDNAs and did not react appreciably with placental cDNA.

Table 1. Screening of CLL cDNA subset library (400 clones) with [^{32}P]cDNA: Classification of clones by intensity of response

Source of [^{32}P]cDNA	Clone type I	II	III	IV
CLL (B cell)	+	+	±	+
CEM (T cell)	+	−	++	++
Lymphoma	+	−	++	+
Placenta	−	−	−	−

Clones selected for further analysis included seven type I, two type II, two type III, and three type IV.

phoma. Filters containing the recombinant clones were screened by hybridization with each [^{32}P]cDNA preparation (Fig. 1). Fourteen clones that reacted positively with cDNA transcripts of poly(A)$^+$RNA from leukemia leukocytes and did not react appreciably with placental cDNA (Table 1) were used in subsequent RNA titration experiments.

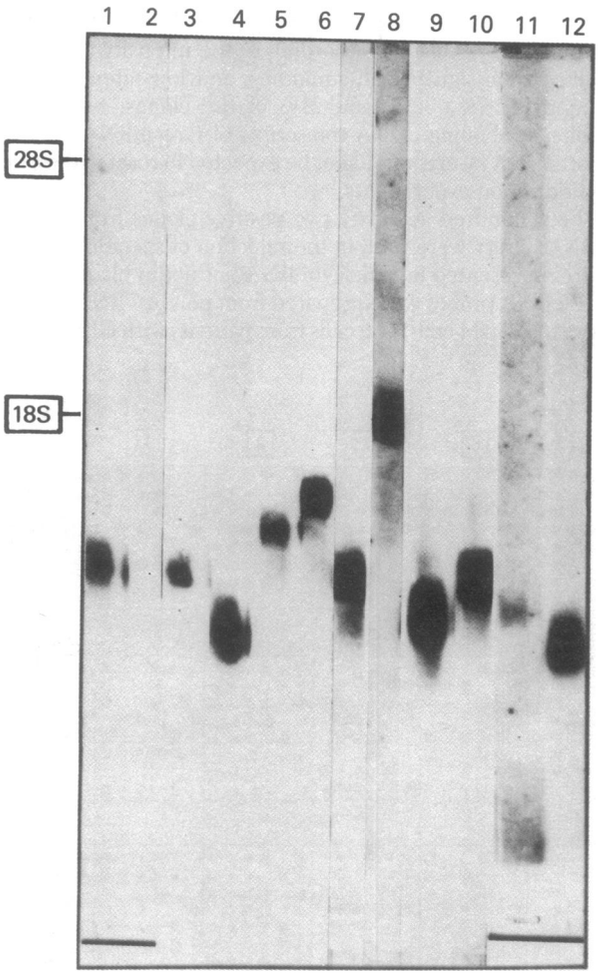

FIG. 2. Reverse Southern blot analysis of poly(A)$^+$RNAs from CLL cells. Aliquots (10 μg) of glyoxal-denatured poly(A)$^+$RNA from CLL cells were subjected to electrophoresis on 1.1% agarose gels in 10 mM phosphate buffer (pH 7.0). The RNA was transferred to nitrocellulose paper, which was cut into strips corresponding to the lanes of the gel. The strips were hybridized separately to nick-translated cloned DNA. Molecular weight markers were 28S and 18S rRNAs and λ phage DNA fragments (cut with *Hin*dIII) run in separate lanes and detected by ethidium bromide staining of the gel. Lanes: 1, clone 9-5A; 2, clone 6-1G; 3, clone 1-3G; 4, clone 1-6G; 5, clone 6-1E; 6, clone 9-2C; 7, clone 5-2C; 8, clone 7-2D; 9, clone 7-3G; 10, clone 5-5G; 11, clone 7-4A; 12, clone 6-4A.

Table 2. Size analysis of cloned cDNA inserts and poly(A)$^+$RNA encoded by cDNA containing recombinant plasmids

Clone	Insert size,* base pairs	Poly(A)$^+$RNA size, bases
5-2C	496	1,060
6-4A	498	800
6-1E	263	1,200
6-2F	154	—
6-1G	154	1,760
1-3G	154	1,080
7-3G	344	910
7-2D	609	1,570
5-5G	456	1,060
7-4A	176	940
9-1C	176	—
1-6G	221	880
8-2C	241	1,320
9-5A	210	1,110

* Calculated by comparison with migration of *Hinf*I restriction endonuclease fragments of pBR322. Inserts contain homopolymer tails of about 14 base pairs at each end.

Two tests were carried out to ensure that the human sequences in the 14 individual plasmids represented different mRNA molecules. Unlabeled denatured poly(A)$^+$RNA from CLL cells was separated by agarose gel electrophoresis and

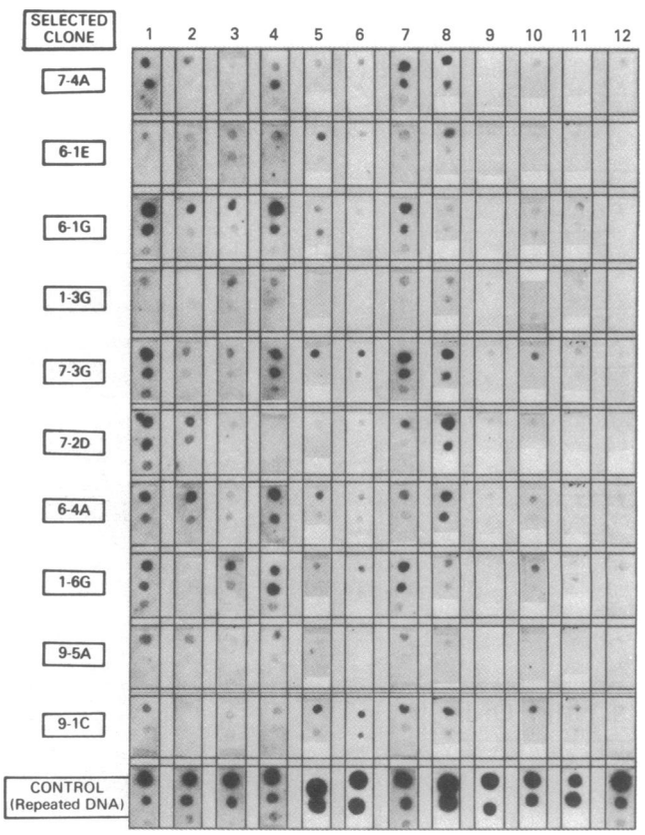

FIG. 3. Dot hybridization of selected clones from the CLL cDNA library with [^{32}P]poly(A)$^+$RNA from various leukemia cells, normal leukocytes, spleen, and placenta. A recombinant clone (BLUR 8) containing a human *Alu* repeat sequence (22) and previously shown (23) to be represented in poly(A)$^+$RNA was used as a control for the end-labeling and hybridization procedure. Lanes: 1, CLL; 2, 13-cell lymphoma; 3, lymphocytic lymphoma; 4, CEM; 5, ALL; 6, AML; 7, CML; 8, blast crisis CML; 9, normal leukocytes; 10, phytohemagglutinin-stimulated leukocytes; 11, spleen; 12, placenta.

transferred to nitrocellulose filters. The resulting immobilized RNA was hybridized with ^{32}P-labeled plasmid DNA. Each plasmid hybridizing to a mRNA of a different size was considered a distinct sequence. Clones 9-5A, 1-3G, 5-2C, and 5-5G hybridize to RNA of similar sizes (Fig. 2) and clones 7-3G and 7-4A also hybridize to RNA of similar sizes.

The different clones were also checked for sequence homology by the Southern blotting technique (21). Each plasmid was digested with restriction endonuclease *Pst* I to release the human cDNA insert, and the DNA fragments were separated by agarose gel electrophoresis. After transfer to nitrocellulose filters, the DNAs were hybridized with single [^{32}P]cDNA inserts. Among the 14 clones examined, only clones 7-4A and 7-3G cross-hybridized, indicating that they share sequence homology. The properties of the recombinant plasmids used in further experiments are summarized in Table 2.

The differential representation of each clone in various kinds of mRNA is shown in Fig. 3. Ten of the 14 clones selected from the cDNA library indicated on the screening grid by dashed circles (Fig. 1) were used in the dot hybridization experiments (the other four clones did not hybridize with any of the [^{32}P]RNA preparations). [^{32}P]mRNA prepared from normal leukocytes, spleen, and placenta did not hybridize significantly with any of the cloned cDNAs. However, RNA from phytohemagglutinin-stimulated lymphocytes hybridized significantly with clones 7-4A, 7-3G, 6-4A, 1-6G, and 9-1C, indicating an increased abundance of these mRNAs in phytohemagglutinin-stimulated cells. Labeled CLL mRNA hybridized strongly with clones 7-4A, 6-1G, 7-3G, 7-2D, 6-4A, and 1-6G and weakly with all other clones. The clones were moderately to highly represented in ALL, CEM, and blast crisis CML mRNAs. RNA hybridizing with clone 7-2D was highly abundant in CLL and blast crisis CML cells. Clone 7-4A was highly represented in CML and blast crisis cells while clone 9-1C was highly represented in ALL, acute myelogenous leukemia (AML), CML and blast crisis CML cells. RNAs hybridizing with clones 6-1E, 1-3G, and 9-5A were only moderately abundant in the various leukemia cells. Consistent with the cross-hybridization experiments, dot hybridization with [^{32}P]mRNA (Fig. 3) indicated that clones 7-4A and 7-3G have similar nucleotide sequences. The intensity of the hybridization signal observed (Fig. 3) was greater with clone 7-3G than with clone 7-4A, as expected with the longer human cDNA insert in clone 7-3G (Table 2).

DISCUSSION

Regulation of gene expression may occur at many levels—RNA transcription, processing, modification, transport, and translation. Substantial evidence exists indicating that the control of gene expression is exerted primarily at the level of transcription (24–27). Recently, Dermen *et al.* (28) used cloned cDNAs to determine the level at which specific rat hepatoma genes were controlled. Their evidence, using both *in vitro*-labeled RNAs from tissue and pulse-labeled cellular RNAs, suggests that the synthesis of most tissue-specific moderately abundant mRNAs is regulated at the level of transcription.

In the experiments presented here, a series of genes that are differentially expressed in human leukemia leukocytes was identified. The methodology used provides a powerful tool to simultaneously monitor the expression of 10–50 genes. The hybridization experiments adequately assigned relative levels of gene expression with much greater sensitivity than methods involving detection of protein products.

Although our cDNA library was prepared from a relatively mature CLL cell population, the differentially expressed sequences identified may reflect expression of genes in immature cells. For example, clone 7-2D is highly expressed in CML blast crisis cells and only moderately expressed in CML cells from a more advanced stage of differentiation. In CML, the blast crisis stage is frequently lymphoblastic rather than myeloblastic in origin.

With a sufficient battery of specific gene probes, one has the potential to simultaneously monitor expression of 50 or more genes and identify changes in gene expression during leukemogenesis. With the exploitation of this approach, it will be possible to further test the hypothesis that the cell differentiation process involves changes in the levels of specific mRNAs.

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