Analysis of differential gene expression by display of 3' end restriction fragments of cDNAs

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ABSTRACT We have developed an approach to study changes in gene expression by selective PCR amplification and display of 3' end restriction fragments of double-stranded cDNAs. This method produces highly consistent and reproducible patterns, can detect almost all mRNAs in a sample, and can resolve hidden differences such as bands that differ in their sequence but comigrate on a gel. Bands corresponding to known cDNAs move to predictable positions on the gel, making this a powerful approach to correlate gel patterns with cDNA data bases. Applying this method, we have examined differences in gene expression patterns during T-cell activation. Of a total of 700 bands that were evaluated in this study, as many as 3–4% represented mRNAs that are upregulated, while ≈2% were down-regulated within 4 hr of activation of Jurkat T cells. These and other results suggest that this approach is suitable for the systematic, expeditious, and nearly exhaustive elucidation of subtle changes in the patterns of gene expression in cells with altered physiologic states.

Identification of genes associated with development and differentiation is an important step for advanced understanding of these phenomena. Early methods developed to identify and clone such genes were primarily based on the principle of subtractive hybridization (1–4). Despite their usefulness, these methods can analyze only a fraction of the overall changes in gene expression, require large amounts of RNA, and are lengthy and laborious. Recently, Liang and Pardee (5) developed a gel-based technique that facilitates a rapid and extensive analysis of differentially expressed mRNAs. Several groups have successfully used this technique to identify differentially expressed genes (6–8). However, certain limitations associated with this technique such as the lack of quantitative correlation with mRNA abundances, a significant incidence of false-positive signals, variable reproducibility of the display patterns, and underrepresentation and redundancy of mRNA signals (6, 9, 10) make it difficult to fully evaluate differential gene expression. More importantly, the size of bands is not always readily predictable from the mRNA sequence.

Amplification of cDNAs at the low primer annealing temperature of 40°C, a nonstringent PCR condition, is considered a major limitation of current gel display protocols (6, 11, 12). Adaptations of the original protocol have been reported in order to overcome some of these limitations such as the use of 1-base anchored oligo(dT) primer for increased representation of mRNAs (9) and the use of long composite primers to achieve reproducible patterns under more stringent PCR conditions (9, 10). However, all these modifications continue to involve annealing of arbitrary primers at 40°C for cDNA amplification in the first few or all PCR cycles.

In this report, we present an alternative approach for cDNA display on gels. Display patterns are generated when restriction enzyme-digested double-stranded cDNA is ligated to an adapter that mediates selective PCR amplification of 3'-end fragments of cDNAs under high-stringency PCR conditions instead of nonstringent arbitrary cDNA amplification. A diversity of patterns is generated by choosing different sets of restriction enzymes and anchored oligo(dT) primers with a heel. Since all cDNAs in a sample acquire a common heel from the oligo(dT) primer during synthesis, most cDNA molecules in a subset [determined by the anchor nucleotides of the oligo(dT)] can be displayed by choosing a combination of restriction enzymes, thus significantly reducing underrepresentation or redundant representation of mRNAs. This approach provides near-quantitative information about the levels of gene expression, can resolve hidden differences in the display gel, produces a single band for each mRNA species, and produces bands of predictable size for known gene sequences. Most importantly, the method produces consistently reproducible display patterns.

In the present study, we examined patterns of RNA expression during early T-cell activation, an extensively studied phenomenon associated with induction of a large number of genes within a relatively short period of time (13, 14). Curiously, there is a limited description of genes that are downregulated upon T-cell activation (15, 16). The present approach offered a convenient method for looking for such products.*

MATERIALS AND METHODS

Stimulation of Jurkat Cells and RNA Isolation. Conditions for growth and activation of Jurkat and peripheral blood T cells, respectively, have been described (16, 17). Total cellular RNA was prepared from untreated and 4-hr phorbol 12-myristate 13-acetate plus phytohemagglutinin-activated Jurkat cells using Trizol reagent (GIBCO/BRL).

cDNA Synthesis and Adapter Ligation. cDNA was synthesized according to the protocol described in the GIBCO/BRL kit for cDNA synthesis. The reaction mixture for first-strand synthesis included 10 μg of total RNA, and 2 pmol of 1 of the 2-base anchored oligo(dT) primers with a heel such as RP5.0 (CTCTCAAGGATCTTACCGCTT$_{18}$AT), RP6.0 (TAATACCGCGCCACATAGCAT$_{18}$CG), or RP9.2 (CAGGGTAGACGACGCTACGCT$_{18}$GA) along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was layered with mineral oil and incubated at 65°C for 7 min followed by 50°C for another 7 min. At this stage, 2 μl of Superscript reverse transcriptase (200 units/μl; GIBCO/BRL) was added quickly and mixed, and the reaction continued for 1 hr at 50°C. Second-strand synthesis was performed at 16°C for 2 hr. At the end of the reaction, the cDNAs were precipitated with ethanol and the yield of cDNA was calculated. In our experiments, ≈200 ng of cDNA was obtained from 10 μg of total RNA. The adapter oligonucle-

Abbreviations: PNK, polynucleotide kinase; IL-2, interleukin 2; RT-PCR, reverse transcription PCR.
*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U38432–U38444).

otide sequences were A1 (TAGCGTCCGGCGCAGCGACG-GCCAG) and A2 (GATCCTGGCCGTCGGCTGTCTGTC-GGCGC). One microgram of oligonucleotide A2 was first phosphorylated at the 5' end using T4 polynucleotide kinase (PNK). After phosphorylation, PNK was heat denatured, and 1 μg of the oligonucleotide A1 was added along with 10× annealing buffer (1 M NaCl/100 mM Tris·HCl, pH 8.0/10 mM EDTA, pH 8.0) in a final vol of 20 μl. This mixture was then heated at 65°C for 10 min followed by slow cooling to room temperature for 30 min, resulting in formation of the Y adapter at a final concentration of 100 ng/μl. About 20 ng of the cDNA was digested with 4 units of *Bgl* II in a final vol of 10 μl for 30 min at 37°C. Two microliters ($\approx$4 ng of digested cDNA) of this reaction mixture was then used for ligation to 100 ng ($\approx$50-fold) of the Y-shaped adapter in a final vol of 5 μl for 16 hr at 15°C. After ligation, the reaction mixture was diluted with water to a final vol of 80 μl (adapter ligated cDNA concentration, $\approx$50 pg/μl) and heated at 65°C for 10 min to denature T4 DNA ligase, and 2-μl aliquots (with $\approx$100 pg of cDNA) were used for PCR.

PCR and Display. The following sets of primers were used for PCR amplification of the adapter ligated 3'-end cDNAs: RP 5.0, RP 6.0, or RP 9.2 was used as 3' primer while A1.1 (TAGCGTCCGG CGCAGCGAC) served as the 5' primer. To detect the PCR products on the display gel, 24 pmol of oligonucleotide A1.1 was 5'-end-labeled using 15 μl of [γ-^{32}P]ATP (Amersham; 3000 Ci/mmol) and PNK in a final volume of 20 μl for 30 min at 37°C. After heat denaturing PNK at 65°C for 20 min, the labeled oligonucleotide was diluted to a final concentration of 2 μM in 80 μl with unlabeled oligonucleotide A1.1. The PCR mixture (20 μl) consisted of 2 μl ($\approx$100 pg) of the template, 2 μl of 10× PCR buffer (100 mM Tris·HCl, pH 8.3/500 mM KCl), 2 μl of 15 mM MgCl$_2$ to yield 1.5 mM final Mg^{2+} concentration optimum in the reaction mixture, 200 μM dNTPs, 200 nM each 5' and 3' PCR primers, and 1 unit of Amplitaq. Primers and dNTPs were added after preheating the reaction mixture containing the rest of the components at 85°C. This "hot start" PCR was done to avoid artefactual amplification arising out of arbitrary annealing of PCR primers at lower temperature during transition from room temperature to 94°C in the first PCR cycle. PCR consisted of 28–30 cycles of 94°C for 30 sec, 56°C for 2 min, and 72°C for 30 sec. A higher number of cycles resulted in smeary gel patterns. PCR products (2.5 μl) were analyzed on a 6% polyacrylamide sequencing gel. For double or multiple digestion following adapter ligation, 13.2 μl of the ligated cDNA sample was digested with a secondary restriction enzyme(s) in a final vol of 20 μl. From this solution, 3 μl was used as template for PCR. This template vol of 3 μl carried $\approx$100 pg of the cDNA and 10 mM MgCl$_2$ (from the 10× enzyme buffer), which diluted to the optimum of 1.5 mM in the final PCR vol of 20 μl. Since Mg^{2+} comes from the restriction enzyme buffer, it was not included in the reaction mixture when amplifying secondarily cut cDNA. Bands were extracted from the display gels as described (7), reamplified using the 5' and 3' primers, and subcloned into pCR-Script with high efficiency using the PCR-Script cloning kit from Stratagene. Plasmids were sequenced by cycle sequencing on an ABI automated sequencer.

RESULTS AND DISCUSSION

The general scheme for our approach is shown in Fig. 1. A 2-base anchored oligo(dT) primer with a heel is used for first-strand cDNA synthesis from total RNA using reverse transcriptase followed by second-strand synthesis by the Gubler–Hoffman method (18). All cDNA molecules thus acquire a common 3' heel. This cDNA is digested with a restriction enzyme and ligated to a Y-shaped adapter similar in principle to the bubble adapter (19). The adapter has an overhang on its 3' end for ligation, and on the 5' end it has a
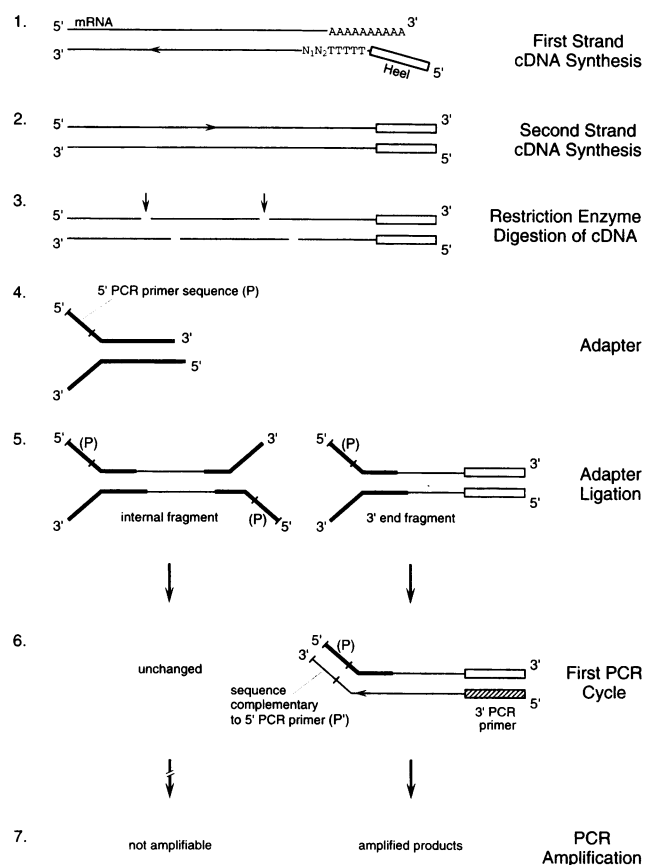

FIG. 1. Schematic of the method for 3'-end cDNA amplification.

stretch of noncomplementary sequence on the opposite strands giving rise to its Y shape (Fig. 1). The 5' PCR primer is made from this Y region (Fig. 1) and therefore cannot anneal to the adapter itself. The upstream fragments of digested cDNA with adapter ligated on both the ends or only one end in the 5'-terminal piece will, therefore, not be PCR amplified. However, the 3' primer anneals to the heel of the 3'-end fragments of cDNA during the first PCR cycle and extends DNA synthesis into the Y region of the ligated adapter, thus synthesizing complementary sequences to which the 5' PCR primer can now anneal. The two PCR primers can then selectively amplify the 3'-end fragments of the cDNA under stringent PCR conditions.

Each 6-base cutting restriction enzyme cuts $\approx$8% of the cDNAs at positions between 50 and 400 bases from the poly(A) tract so that more than 12 6-base cutters will be needed to approach complete representation of cDNAs, each being used with several different anchored oligo(dT) primers. Also, we estimate that, at best, 100–150 discrete bands can be detected in a single gel lane. Therefore, at least 100 lanes need to be run under different conditions to study the overall pattern of gene expression in any single cell type.

A major advantage of the present approach is that the size of a known cDNA product and hence its position on the display gel is predictable. Interleukin 2 (IL-2) is a well studied cytokine expressed only in activated but not in resting T cells (13) and should be displayed as a band of predictable size. To confirm this and to test the ability of the method to display differences, we made cDNA from resting and 4-hr activated human peripheral blood T-lymphocyte RNA using oligo(dT) primer RP 5.0, which has a heel and 3' anchor residues 3'-TA-5' complementary to the 5'-AT-3' dinucleotide in the IL-2 mRNA sequence immediately preceding the poly(A) tail (for details, see ref. 20). Restriction digestion with *Bst*Y1 should produce a 146-bp 3'-end fragment of IL-2 cDNA (21).

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When added to the sizes of the 3' oligo(dT) and 5' adapter, this should produce a band of 209 bp on the display gel. A distinct band of the predicted size was produced in the activated but not in the resting T-cell sample on display of *Bst*Y1 cut cDNA (Fig. 2, lane 2). Sequencing of this fragment confirmed the presence of the 5' adapter followed by a *Bst*Y1 site, IL-2 3'-end sequences, the polyadenylylation signal AATAAA, the downstream oligo(A) tract, and the heel primer (data not shown).

We then compared RNA from resting and 4-hr activated Jurkat cells. We initially displayed 3' cDNA fragments produced by restriction enzymes *Bgl* II, *Bcl* I (data not shown), and *Bam*HI as they produce GATC overhangs compatible with the same adapter. These enzymes produced different display patterns with the same pool of cDNAs (Fig. 3B). Moreover, these patterns were consistently reproducible in several different sets of experimental conditions described in the legend to Fig. 3. The reproducibility of the method was also illustrated by the large number of common bands between untreated and activated Jurkat cDNAs (Figs. 2 and 3).

To examine the validity of differences seen on the display gels, bands were subcloned and sequenced, and a specific pair of oligonucleotide primers based on these sequences was used for reverse transcription PCR (RT-PCR) (17) with total RNA from untreated and activated normal T cells and Jurkat cells. Two differences in *Bgl* II-digested cDNA (Fig. 3C, see arrows pointing to lanes 1 and 2; see also Fig. 4) were verified to be true.
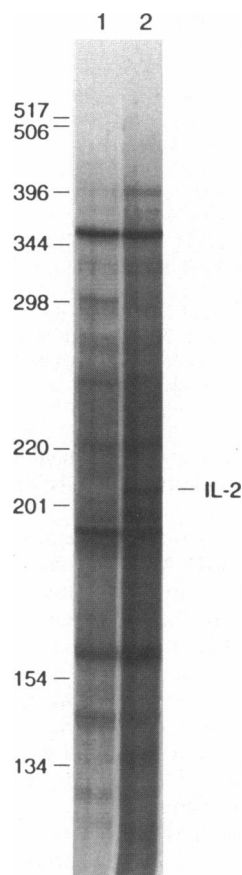

FIG. 2. Predictability of the band size of known cDNAs on the display gel. Display of *Bst*Y1-digested cDNA prepared from resting and activated T cells produces the predicted 209-bp band corresponding to IL-2 mRNA 3'-end sequence in activated T cells (lane 2) but not in resting T cells (lane 1). After extracting the band from the gel, it was confirmed to be IL-2 by sequencing. Before PCR amplification, the adapter ligated cDNA was recut with *Alu* 1, *Rsa* I, and *Msp* 1 since without recutting smeary patterns resulted, as *Bst*Y1 is a more frequently cutting restriction enzyme. Numbers on left are bp.

Overlapping bands that mask the true differences can be resolved on recutting the adapter ligated 3'-end cDNA fragments (shown in step 5 of Fig. 1) before PCR amplification. If a site for a restriction enzyme used for recutting is present in one of the two comigrating bands, it will be cut into one part with an adapter and the other with a heel, which cannot be PCR amplified and will therefore be eliminated. Using this approach, we successfully uncovered additional differences between untreated and 4-hr activated Jurkat cells when *Bgl* II-cut and adapter ligated cDNA was further digested with *Hin*fI (Fig. 3C, see arrows pointing to lane 4; see also Fig. 4).

The choice of enzymes for primary or secondary cutting was arbitrary although a combination of more than one enzyme can be used for recutting. Another advantage of recutting is that recovery of low-abundance cDNAs is enhanced because removal of high-abundance bands by recutting allows access of these fragments to PCR primers (Fig. 3C, lanes 3 and 4). In addition, recutting can be used to minimize redundancy between fragments in different lanes. For example, *Bgl* II-cut cDNA fragments can be recut with *Bam*HI and vice versa, so that the two samples share no amplified products. Confirmation that a band corresponds to a known cDNA sequence can be obtained by recutting the cDNA with appropriate restriction enzymes prior to amplification. A large number of variations of the display patterns can therefore be produced in this method to look for differentially expressed genes by (*i*) a combination of a number of different 2-base anchored oligo(dT) primers with a heel for making cDNAs, (*ii*) a number of different restriction enzymes that can be used for primary cutting of these cDNAs and, (*iii*) the number of restriction enzymes used for secondary cutting for each primary cut.

Consistent with the reproducibility of the gel patterns, most differences in the intensity of cDNA amplification products corresponded to differences in mRNA levels. A total of 16 bands were subcloned, sequenced, and examined by RT-PCR. Of these, 15 showed changes in levels of expression predicted from the gel pattern (Fig. 4). Of the 15 sequences, 1 was c-myc transcription factor Puf (22), 1 was IL-2 (21), and the remainder were novel sequences not represented in the data bases. Each product contained a polyadenylation signal upstream of the oligo(dT) tract. At the other end of each product, the expected 4-base overhang of the adapter was seen, followed by the base predicted from the specificity of the enzyme used for the initial cutting of the cDNA. For example, bands from display patterns generated with the enzyme *Bgl* II all had the sequence of the adaptor including the GATC of the adapter overhang, followed by a T that would be expected if the 5' end of the attached cDNA had been generated by *Bgl* II. In every instance, bands recovered from the gel lacked internal cleavage sites for the restriction enzymes used for primary or secondary cutting. The levels of PCR products of the 12 induced and 1 down-regulated cDNAs and presumably their mRNAs vary with respect to IL-2 and β-actin (Fig. 4). Therefore, while it is easier to detect and isolate cDNA fragments corresponding to the more abundant mRNAs, this approach allows us to detect and subclone samples from quite rare mRNAs.

One of the most important factors affecting clarity of the display patterns is quantity of the template and the number of PCR cycles. We found that ≈100 pg of cDNA template is appropriate for PCR amplification. However, before attempting to analyze differences in display gels, we routinely amplified serial dilutions of the template to choose a concentration that will produce clear and reproducible patterns in 28–30 PCR cycles. High template concentrations produced smeary patterns, while at very low template concentration bands started to drop out, producing artefactual differences on the gel. Good quality of RNA is also a prerequisite for clean display patterns. Replicates of PCR amplified samples were run on the gel to look for the consistency in the difference of
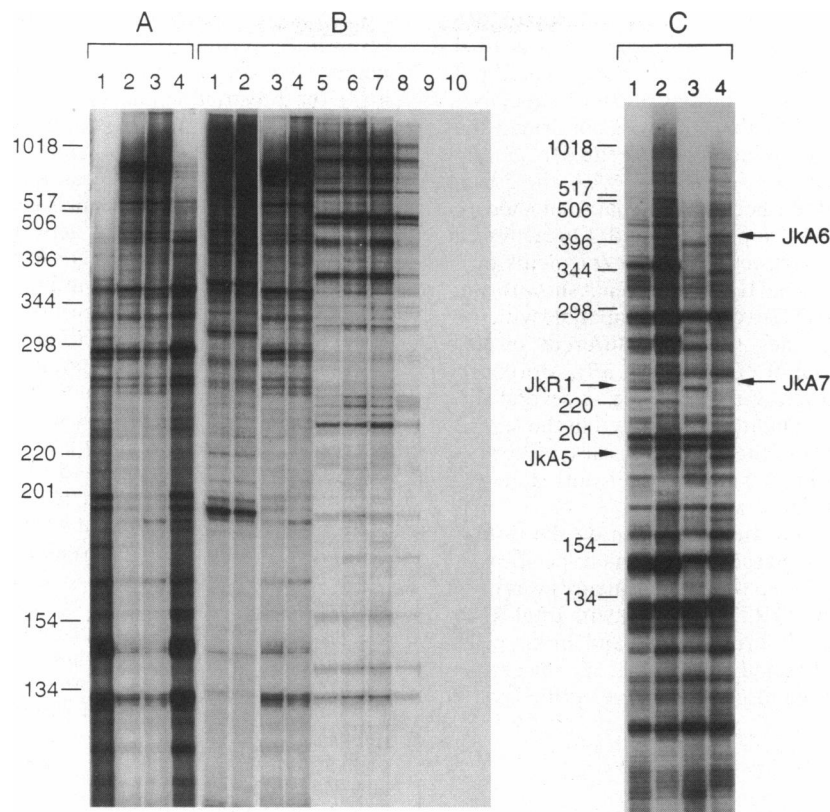
FIG. 3. Reproducibility of display patterns and their diversity generated by different restriction enzymes and anchors in oligo(dT) primers with a heel. (*A*) Reproducible patterns were observed on display of *Bgl* II-digested cDNAs prepared by using oligo(dT) primer RP 6.0 from untreated (lanes 1 and 2) and activated (lanes 3 and 4) Jurkat RNA samples isolated in two separate experiments. (*B*) Lanes 1, 3, 5, 7, and 9, cDNA samples from untreated cells; lanes 2, 4, 6, 8, and 10, cDNA samples from activated Jurkat cells. Different display patterns were produced on *Bgl* II digestion of cDNAs prepared by using different anchor bases (RP 9.2 in lanes 1 and 2; RP 6.0 in lanes 3 and 4). However, digestion of the RP 6.0 cDNAs with different restriction enzymes produced different display patterns (*Bgl* II in lanes 3 and 4 and *Bam*HI in lanes 5 and 6). Aliquots of RP 6.0 cDNAs digested with *Bam*HI in separate experiments showed consistent patterns between untreated (lanes 5 and 7) and activated (lanes 6 and 8) Jurkat cells when ligated to adapter and PCR amplified. Separate adapter ligations to aliquots of the same batch of restriction enzyme-digested cDNAs, as expected, showed identical patterns (data not shown). However, in the absence of adapter ligations no pattern was generated (lanes 9 and 10). All samples in *A* and *B* were run on the same gel; however, lanes 1–4 in *A* and lanes 1, 2, and 3–10 in *B* were run in adjacent lanes on the gel. (*C*) RP 6.0 cDNA from untreated (lanes 1 and 3) and activated (lanes 2 and 4) Jurkat cells was displayed upon digestion with *Bgl* II (lanes 1 and 2), while in lanes 3 and 4 *Bgl* II-digested and adapter ligated cDNA was redigested with restriction enzyme *Hin*fI before PCR amplification. Arrows point to bands revealed as true differences on recutting (see text and Fig. 4). For detailed display protocols, see *Materials and Methods*. Numbers on left are bp.

intensity of the band under consideration and unligated cDNAs were PCR amplified as controls. We found that subcloning a band of interest is better than direct analysis of the reamplified band recovered from the gel, and it is necessary to determine whether the recovered band is of the correct size.

Increasing the number of adapter ligated 3'-end cDNA fragments in a sample can cause blurred patterns. *Sau*3A1 (a 4-base cutter) produced smeary patterns, although the same amount of cDNA digested with *Bgl* II or *Bam*HI (6-base cutters) produced clear gel patterns. *Sau*3A1 being a frequent cutter produces more amplifiable 3' ends from a cDNA population, which then crowd together on the gel. RNA primed with oligo(dT) primers containing a mixture of bases in the subterminal anchor position produced crowded patterns because a larger number of cDNA molecules are synthesized.

The extent to which anchored oligo(dT) primers prime cDNA synthesis from mRNAs whose sequence does not match the anchor bases perfectly has been a major limitation of cDNA display methods. We tried a number of conditions to enhance specificity of this priming and found that primer extension by reverse transcriptase at 50°C was optimal. However, even under these optimized conditions some known cDNA products arose because of mispairing or looping out of the subterminal anchor base (data not shown). Nevertheless,

different yet consistent and reproducible patterns were obtained with cDNAs made from oligo(dT) primers with different anchor bases.

Overall, from four pairs of lanes representing untreated and activated Jurkat cell mRNAs, ≈700 bands could be evaluated. Of these ≈4% appeared to represent species that increase on activation and as many as 2% significantly decreased on activation. T-cell activation is an extensively studied phenomenon and ≈80 genes whose expression is increased in the early phase of activation have been recognized (13). In the present experiments, the estimated number of 3'-end mRNA sequences whose expression is altered within 4 hr after T-cell activation is an order of magnitude higher than might be presumed from earlier studies (2–4).

One cDNA, JkR1, that was down-regulated on activation in both peripheral blood T cells and Jurkat cells was chosen for further study. Down-regulation of JkR1 in peripheral blood T cells was evident 4 hr after addition of ionomycin and diacylglycerol. When resting T cells were exposed to actinomycin D, JkR1 mRNA was relatively stable for up to 3 hr (data not shown). This raises the possibility that part of the down-regulation might be achieved by mRNA destabilization. Schneider *et al.* (23) have studied a small group of GAS genes whose mRNA levels decline on refeeding cells with serum, and several of these mRNAs were also down-regulated by desta-

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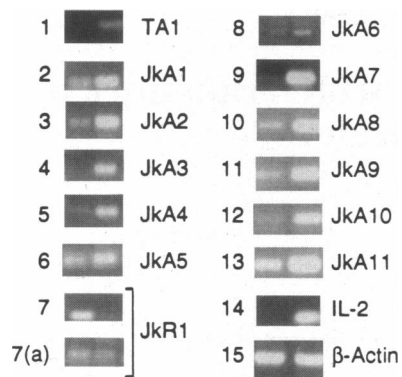*Proc. Natl. Acad. Sci. USA* 93 (1996)   663

FIG. 4. Analysis of differences from display gel by RT-PCR of original total RNA sample. In each of the panels 1–15 the left lane represents untreated while the right lane represents activated Jurkat cell cDNA. (However, in panel 7 but not in panel 7a peripheral blood T-cell RNA was used for RT-PCR.) Bands of interest were extracted from the display gel, subcloned, and sequenced, and specific PCR primers were made. The method to prepare RNA and RT-PCR has been described (17). Briefly, 1 μg of total RNA was reverse transcribed at 37°C using 100 ng of random hexamer primer in a total vol of 20 μl. After heat inactivation of reverse transcriptase, the final volume of the reaction mixture was adjusted to 50 μl with water. Of this diluted sample, 2 μl was taken for PCR amplification in a final vol of 50 μl containing 5 μl of 10× PCR buffer (100 mM Tris·HCl/500 mM KCl), 200 nM each 5' and 3' primers, 200 μM dNTPs, 1 unit of Amplitaq, and 1.5 mM MgCl$_2$. PCR consisted of 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec. PCR samples were analyzed on 1.5% agarose gel and stained with ethidium bromide. Samples in panels 1–15 were analyzed in separate experiments; however, IL-2 and β-actin always showed the same patterns in each experiment as in panels 14 and 15, respectively.

bilization (20). JKR1, however, is different from the GAS genes both in sequence and in the fact that the level of its mRNA was not decreased simply by refeeding but only after specific activation of T cells.

Strikingly, there are very few references in the literature to genes whose expression is down-regulated on T-cell activation (15, 16). In addition, a gene with a zinc finger motif, cloned in our laboratory, is also down-regulated in 4-hr activated Jurkat and peripheral blood T cells (K. Prakash and S.M.W., unpublished results). The present results suggest that changes in the intracellular environment upon T-cell activation are a combined result of down-regulation of a set of genes in addition to induction of gene expression.

We have shown the ability of the present method to uncover multiple differences between rather similar samples of untreated and 4-hr-activated Jurkat cells. The reproducibility and extensive representation of mRNAs obtained with this method allow a systematic analysis of a sample, taking full advantage of sequence data bases. Since the lane and size of the band of a known gene can be easily predicted, gel patterns can be used to evaluate changes in the level of expression of known mRNAs prior to further analysis. As a large fraction of cDNAs become represented in the data bases as 3' expressed sequence tags (ESTs), one can use data base searches to limit or define candidate genes corresponding to any band whose abundance changes. This will be an increasingly powerful approach as more cDNA and genomic sequences accumulate. We are also exploring an approach to display internal restriction fragments of cDNAs by using either one adapter to display fragments produced by one restriction enzyme or two separate Y-shaped adapters for the fragments with two different restriction sites at their ends. This should allow unmasking of the differentially expressed cDNAs from others that share a common 3' un-

translated sequence and hence could not be differentiated by displaying 3'-end cDNA fragments. Alternative spliced forms of an mRNA producing different sized internal fragments could also be analyzed. Furthermore, by using fluoresceinated primers, automated analysis of the control and test samples may be possible. And finally, the use of two different restriction sites, one within the adapter and the other within the heel primer, can expedite and orient the cloning. Because of its enhanced sensitivity this approach could be used to study the time course of appearance or disappearance of a set of mRNAs in a variety of eukaryotic systems and extrapolate back to shorter time intervals after initiating the stimulus.

Note. While the present manuscript was under review, an alternative technique to display restriction endonuclease fragments near the 3' end of cDNAs was described (24).

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