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# Multiplex messenger assay: simultaneous, quantitative measurement of expression of many genes in the context of T cell activation

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## ABSTRACT

**The hybridization signature approach, using colony filters and labeled complex probes, can provide high throughput measurement of gene activity. We describe here the implementation of this method to follow the expression levels of 47 genes in resting and activated T cells, as well as in epithelial cells. Using 4-fold spotting of colonies, imaging plate detection and various correction and normalization procedures, the technique is sensitive enough to quantify expression levels for sequences present at 0.005% abundance in the probe. Comparison with Northern blotting shows good consistency between the two methods. Upon activation of a T cell clone by an anti-CD3 antibody variations ranging from 2- to 20-fold are measured, some of which had not been reported previously. This 'multiplex messenger assay' method, performed using available commercial apparatus, can be used in many cases where simultaneous assessment of mRNA levels for many genes is of interest.**

## INTRODUCTION

Gene indexes with many thousands of entries have been constructed by tag sequencing of randomly selected cDNA clones (1–8) and are widely available in repositories such as the db EST database (9). As more and more genes are identified, efforts are redirected towards understanding the control of gene expression that occurs in a strictly ordered time- and cell-dependent fashion. In order to analyze the expression profiles of a large number of known genes in the tissue (or cell) of interest we have adapted a large scale gene expression analysis system recently described by us (10). It is based on the hybridization of complex probes with high density colony cDNA filters followed by quantitative measurement of the amount of hybridized probe on each colony. A somewhat similar approach using PCR products of *Arabidopsis thaliana* cDNAs has recently been reported (11).

Our method, called MMA, for multiplex messenger assay, is applied to the investigation of differential expression of a set of known T cell genes in three cell types: a thymic epithelial cell line, a cytotoxic T cell clone and the same T cell clone stimulated by an anti-CD3 antibody. The activation of T lymphocytes by antigen during an immune response is mediated by the T cell receptor, which recognizes peptide antigens bound to self major histocompatibility complex (MHC) molecules on the surface of an antigen-presenting cell. This stimulation initiates a cascade of biochemical events that culminate in cellular differentiation and proliferation (12). The activation by an anti-CD3 antibody simulates the events observed after activation. The effect of the mechanisms used by cells at the transcriptional level to regulate the numerous genes involved in activation (including alterations of transcriptional rate, termination of transcription and mRNA stability) are quantified in a single step by our method.

## MATERIALS AND METHODS

### Cell lines

KB5.C20, a CTL clone of B10.BR origin specific for the H-2K$^b$ alloantigen, was maintained in long-term culture as described (13). Samples of $20 \times 10^6$ cells were grown in RMPI medium with 10% FCS alone or in the presence of plastic coated anti-CD3ε (145.2c.11) mAb (14) for 3 h.

The MTE-1D epithelial cell line, obtained after MTE cell line subcloning as described (15), was grown in standard DME with 20% FCS.

### Selection and spotting of clones corresponding to known genes

Most of the cDNA clones used were obtained from an adult mouse thymus cDNA library (10) by hybridization of filters containing part of the library with probes corresponding to known genes. Others were found among already sequenced clones from the same library. For some additional clones (including the control *A.thaliana* cytochrome c554 gene) the cDNA insert was transferred from the original cloning vector to that used for the cDNA library (pcDNA1) and then transformed into MC1061 p3 bacteria to obtain a coherent set of clones in the same plasmid vector and bacterium. Three clones

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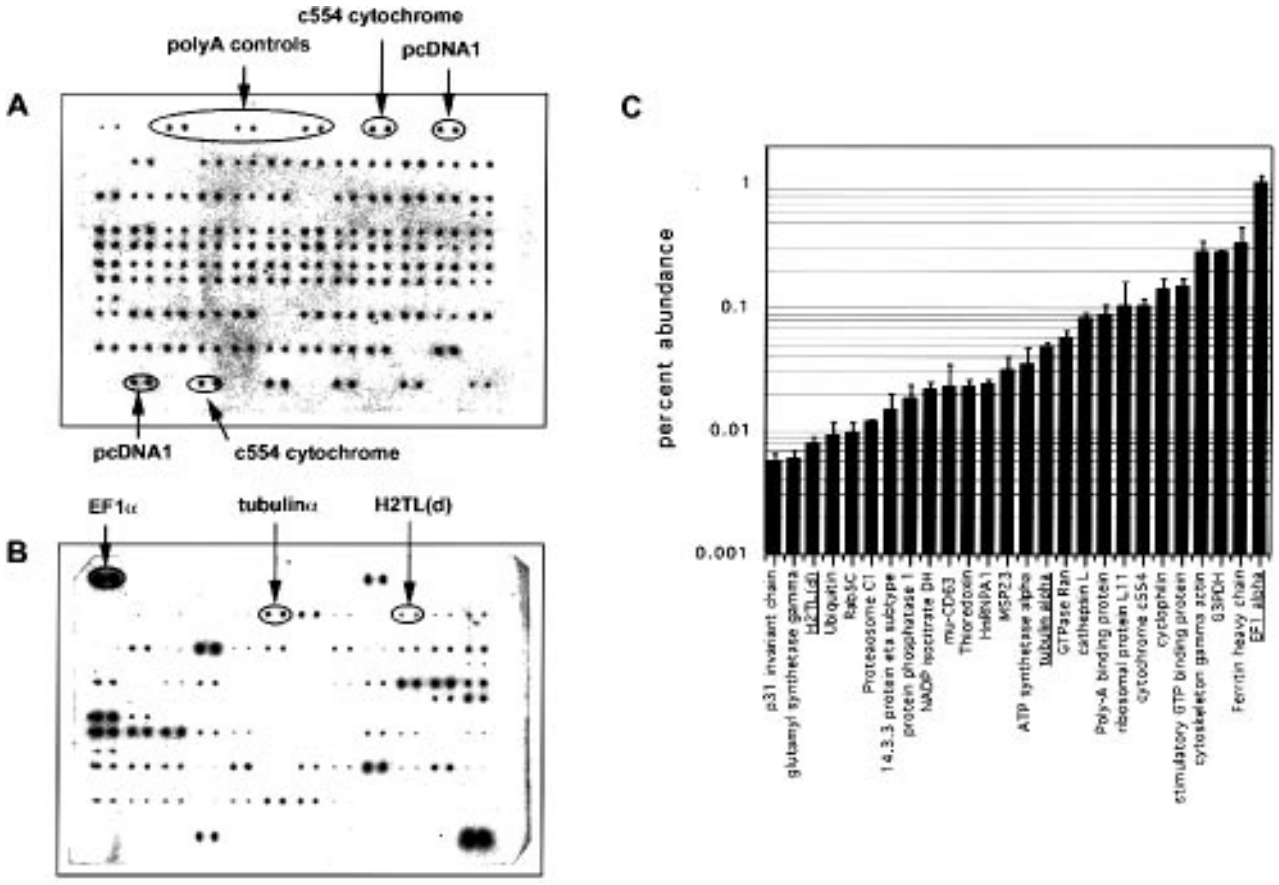


**Figure 1.** MMA colony filter hybridized with the vector oligonucleotide probe (**A**) and with a complex probe made from 25 µg total RNA of the MTE-1D cell line (**B**). Each colony has been spotted in duplicate twice in two opposite symmetrical areas of the filter. The diagram (**C**) shows the results for successfully quantified colonies; all the genes are ordered by increasing intensity on a logarithmic scale in percentage of abundance relative to cytochrome c554, present at 0.1% (see Results). The intensities correspond to the average values of the four spots corrected by vector hybridization and the error bars to the difference between the maximum and the minimum values measured for the spots.

containing essentially a poly(A) sequence (50, 60 and 90 bp) were obtained by appropriate digestion of the poly(A) tail of sequenced cDNAs followed by cloning at the multiple cloning site of the pcDNA1 vector. In all cases participation of the original insert in addition to the poly(A) stretch is <20 bp.

Filters were prepared using a BIOMEK 1000 (Beckman) robotics workstation and a 96 pin tool. Colonies from freshly grown replica plates were spotted onto Hybond N filters (Amersham) (10). Each colony was spotted in quadruplicate twice in two opposite symmetrical areas of the filter (see Fig. 1A). Filters were subsequently treated as described by Nizetic (16).

## Preparation of the *A.thaliana* cytochrome c554 messenger RNA

The *A.thaliana* cytochrome c554 was provided in the pHD-1 vector by Herman Hofte (INRA, Versailles, France).The messenger RNA of this gene was prepared from this cDNA cloned into Bluescript SK+ vector at the *Not*I restriction site and messenger RNA was synthesized from the T3 promotor using the RiboMax large scale production system (Promega).

## Preparation and labeling of complex probes from total RNA and hybridization conditions

Total RNA was isolated from cell lines using the Trizol reagent (Gibco BRL).

Complex probes were prepared from total RNA with an excess of oligo(dT) (25) to saturate the poly(A) tails and ensure that the reverse transcribed product does not contain long poly(T) sequences. Aliquots of 25 µg total RNA, 8 µg $dT_{25}$ plus 300 ng $dT_{12–18}$ and a defined amount (0.5–5 ng in different experiments) of cytochrome c554 mRNA were mixed, heated to 70°C to remove secondary structure in the RNA and progressively cooled to 43°C to ensure annealing of oligo(dT) with the poly(A) tail. Complex probes were then prepared in 25 µl by simultaneous reverse transcription and labeling for 1 h at 43°C in the presence of 50 µCi [$^{32}$P]dCTP, 5 µM dCTP, 0.8 mM each dATP, dTTP and dGTP and 200 U RNase H reverse transcriptase (Gibco BRL). RNA is removed by treatment at 68°C for 30 min with 1 µl 1% SDS, 1 µl 0.5 M EDTA, 3 µl 3 M NaOH and then equilibrated at room temperature for 15 min. Neutralization is with 10 µl 1 M Tris–HCl plus 3 µl 2 N HCl. Unincorporated nucleotides were removed by purification on a G50 column. The probe (after 5 min denaturation at 100°C) was

then incubated with 2 µg poly(dA) ($dA_{80}$) in 1 ml hybridization mix (5× SSC, 5× Denhart's, 0.5% SDS) for 2 h at 65°C. Pre-hybridization and hybridization were both performed for 20 h. After hybridization filters were washed in 2× SSC, 0.1% SDS for 20 min and twice in 0.2× SSC, 0.1% SDS at 65°C for 1 h.

### Vector oligomer labeling and hybridization conditions

Hybridization of oligomers (labeled at the 5′-end with [$\gamma$-$^{32}$P]ATP and kinase) was in 6× SSC, 5× Denhardt's mix, 1% SDS for 15 h at 42°C, followed by two short (2 min) washes in 6× SSC, 0.1% SDS at room temperature. The vector oligomer sequence used was 5′-GCTTATCGAAATTAATACGACTCACTATAG-3′.

### Measurement of hybridization signals

Quantitative data were obtained using an imaging plate device. The hybridized filter was exposed to an imaging plate for 20–35 h and then scanned in a Fujix Bas 1000 (Fuji) system. Hybridization signatures were determined with a modified version of the Bioimage software (Millipore) running on a Unix workstation (17). The resulting quantified data were then analyzed on a microcomputer (Macintosh Centris 650) using Excel software with macro commands that compute average values for each colony.

### Northern blot analysis and quantification

Northern blot analysis was performed according to Maniatis *et al.* (18) with nylon membranes (Hybond N; Amersham). The same amount of KB5.C20 and MTE-1D total RNA (25 µg) or serial dilutions (26, 13, 8.6, 6.5, 5.2 and 2.6 µg) for sensitivity estimations were loaded on the gels. The resulting Northern blots were hybridized with probes labeled by random priming with [$\alpha$-$^{32}$P]dCTP using purified cDNA inserts (19). Northern blots were exposed to a Fuji imaging plate and to X-ray film and the hybridization signals were quantified using Fujix Bas 1000 software.

## RESULTS

The first section describes the MMA filter and the hybridization conditions for a complex probe prepared from total RNA. Controls are detailed in the three following sections, mainly the standardization provided by a plasmid containing an *A.thaliana* cytochrome gene that does not hybridize with mouse sequences, and an evaluation of the sensitivity and reproducibility of signal intensity measurements. The two final sections present results on differential expression of a set of genes between two different cell types, an epithelial cell line (MTE-1D) and a resting cytotoxic T cell clone (KB5.C20), and same T cell clone in a resting or activated state.

### MMA filters and hybridization results

In the experiments reported here we used a filter containing 47 clones corresponding to a series of known genes (Table 2), spotted in quadruplicate to improve the precision of the measurement. The filter was hybridized successively with a vector probe (Fig. 1A) and a complex probe (Fig. 1B). This was prepared with total RNA from the MTE-1D cell line containing as an internal standard a small amount of *in vitro* transcribed RNA of *A.thaliana* cytochrome c554 corresponding to an approximate abundance of 0.1% (see below). Artefactual hybridization, via the poly(A) stretch present in some clones, can be a significant problem, as already discussed (10,20). To avoid production of poly(T) tracts the probe was prepared by simultaneous reverse transcription and labeling in the presence of [$^{32}$P]dCTP, using a large excess of oligo(dT) primers ($dT_{25}$) under stringent hybridization conditions (see Materials and Methods). Three plasmids containing only poly(A) sequences were used as negative controls, for which, as for the vector control, the signal must be close to the filter background. This checks for elimination of this artefact.

Hybridization with the vector probe allowed detection of all the colonies that had grown (Fig. 1A). With the complex probe (Fig. 1B) approximately half of the colonies gave hybridization signals; after quantification the intensity values ranged over more than two orders of magnitude. Three sets of spots are indicated on the image obtained with the complex probe (Fig. 1B), corresponding to three levels in the diagram. Figure 1C shows a graphical representation of the data obtained after imaging plate exposure and quantification. Each hybridization signal was corrected by dividing by the signal obtained after vector hybridization and normalized using the

**Table 2.** Clones used to produce the MMA-1 filter

| spotname | mouse cDNA | source |
|---|---|---|
| 000A0101 | EF1 alpha | MTA |
| 000A0301 | poly A 50pb | MTA |
| 000A0501 | poly A 60pb | MTA |
| 000A0701 | poly A 90pb | MTA |
| 000A0901 | cytochrome c554 | Herman Hofte |
| 000A1101 | PcDNA1 | Invitrogen |
| 000B0201 | CD3 epsilon | CIML (Bernard Malissen) |
| 000B0401 | CD8 alpha | CIML (Eric Vivier) |
| 000B0501 | p59-FYN | CIML (Eric Vivier) |
| 000B0601 | tubulin alpha | Christiane Bouchier |
| 000B0701 | cathepsin L | MTA |
| 000B0801 | Ubiquitin like protein | MTA |
| 000B0901 | mu-Rho | MTA |
| 000B1001 | H2TL(d) | MTA |
| 000B1101 | Proteaosome C1 | MTA |
| 000B1201 | protein phosphatase 1 | MTA |
| 000C0101 | EN-7 | MTA |
| 000C0201 | GTPase Ran | MTA |
| 000C0301 | HMG2 protein | MTA |
| 000C0401 | G3PDH | MTA |
| 000C0501 | Rab5C | MTA |
| 000C0601 | NADP isocitrate DH | MTA |
| 000C0801 | MSP23 | MTA |
| 000C1001 | 14.3.3 protein eta subtype | MTA |
| 000C1101 | mu-CD63 | MTA |
| 000C1201 | ribosomal protein L11 | MTA |
| 000D0101 | Poly-A binding protein | MTA |
| 000D0301 | glutamyl synthetase gamma | MTA |
| 000D0401 | HnRNPA1 | MTA |
| 000D0501 | CD3 delta | CIML (Bernard Malissen) |
| 000D0701 | ZAP-70 | MTA |
| 000D0801 | p31 invariant chain | CIML (Jean Davoust) |
| 000D0901 | ATP synthetase alpha | MTA |
| 000D1001 | Ferritin heavy chain | MTA |
| 000D1101 | cytoskeleton gamma actin | MTA |
| 000D1201 | stimulatory GTP binding protein | MTA |
| 000E0101 | cyclophilin | MTA |
| 000E0201 | Thioredoxin | MTA |
| 000E0301 | interferon gamma | CIML (A.M. Schmitt-Verhulst) |
| 000E0401 | CTLA-1 | CIML (Pierre Golstein) |
| 000E0501 | CTLA-3 | CIML (Pierre Golstein) |
| 000E0601 | CD4 | CIML (Bernard Malissen) |
| 000E1201 | IL2 receptor alpha | CIML (A.M. Schmitt-Verhulst) |
| 000F0101 | perforin | CIML (Pierre Golstein) |

From left to right: position of the clone on the MMA-1 plate; name of the gene and origin of the clone. Clones indicated as MTA (for mouse thymus adult) were obtained from our library by tag sequence identification or hybridization. Clones indicated as CIML XXX were provided by the investigator indicated in our Institute and re-cloned in pcDNA1 as described in Materials and Methods. Other clones were provided by outside investigators whose work is cited in the reference list.

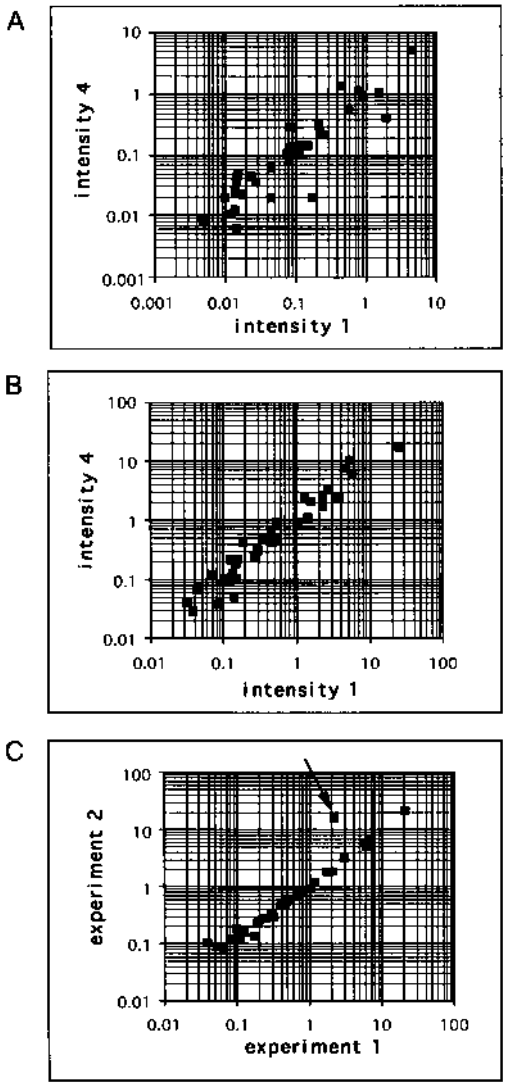


**Figure 2.** Data improvement by vector correction and by averaging. (**A**) The signals from spots 1 and 4 are compared for the whole set of clones. (**B**) Improvement of the reproducibility after vector correction. (**C**) The results of two independent hybridizations using RNA from the same cell line with different amounts of control RNA are compared, taking the average of all four spots after vector correction. The clone shown by an arrow in (C) corresponds to the cytochrome c554 control; in one hybridization 0.5 ng of *in vitro* transcribed RNA from this clone had been added, 5 ng in the other.

c554 signal (see below). Results are ordered by increasing hybridization signal on a logarithmic scale. The hybridization intensity of EF1-α corresponds to an abundance of 1%, the abundance of α tubulin is between 0.01 and 0.1% and H2TL(d) is among the lowest intensities, corresponding to an abundance of <0.01%.

## Use of an *A.thaliana* cytochrome c sequence as an external standard

To standardize hybridization intensities obtained in several experiments we used the *A.thaliana* cytochrome c554 cDNA sequence (1 kb insert), which has no homology with mammalian DNA. To compare independent hybridizations more precisely, the same amount of c554 RNA, *in vitro* transcribed with T3 polymerase from the corresponding cDNA clone, was added before labeling to the total RNA of each cell type or tissue to be tested. The quantification of corresponding colonies present on each filter (Figs 1A and B and 5A and B) allowed us to normalize each independent hybridization according to this value, which corrects for differences in the labeling, washing, duration of exposure and progressive degradation of the filters. These variations can be taken into account so that the differential expression levels for each clone can be compared with greater confidence.

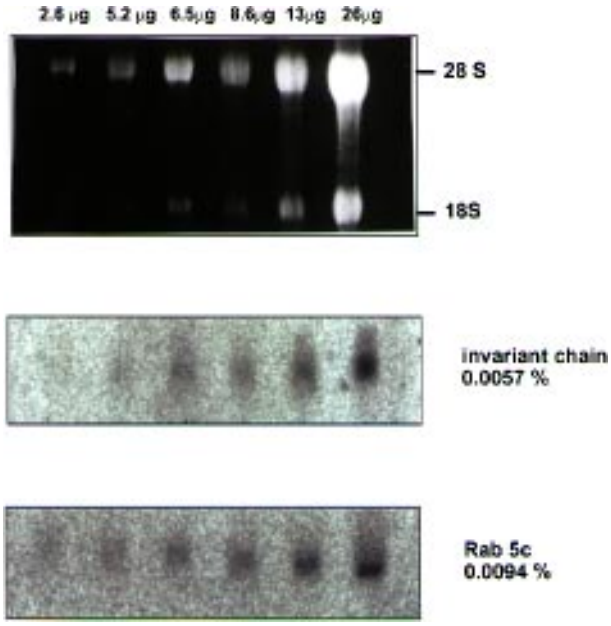


**Figure 3.** Estimation of the detection levels by Northern blotting. A gel was loaded with MTE-1D total RNA. Right to left: 26 µg (1/1); 13 µg (1/2); 8.6 µg (1/3); 6.5 µg (1/4); 5.2 µg (1/5); 2.6 µg (1/10). (Top) RNA visualized by UV after ethidium bromide staining. The resulting Northern blot was hybridized with the invariant chain p31 (middle) and Rab 5c (bottom) probes. These two clones show an abundance of 0.0057 and 0.0094% respectively with a complex probe prepared from MTE-1D.

The *A.thaliana* clone also provided a direct measurement of sensitivity. Increasing quantities of c554 RNA corresponding to abundances of 0.01, 0.1 and 1% with respect to poly(A)$^+$ RNA (assumed to represent 2% of the total RNA) were added to the total RNA before labeling. We observed that the signal intensities were proportional to the amount of c554 RNA in the probe (data not shown). In addition, we reproducibly detected a number of signals on other clones with an intensity less than half that observed on the *A.thaliana* clone when the corresponding sequence was provided at the 0.01% level. Reproducibility was satisfactory down to half this value and we conclude from these measurements that our limit of detection is ~0.005% abundance (which corresponds to an abundance of 1/20 000). Since the reproducibility decreases drastically below 0.005%, we chose to analyze only genes whose signals fell above this threshold.

## Validity and reproducibility of signal intensity measurement

Previous analysis indicated that, under our hybridization conditions, the signal intensity is proportional to both the amount of target and

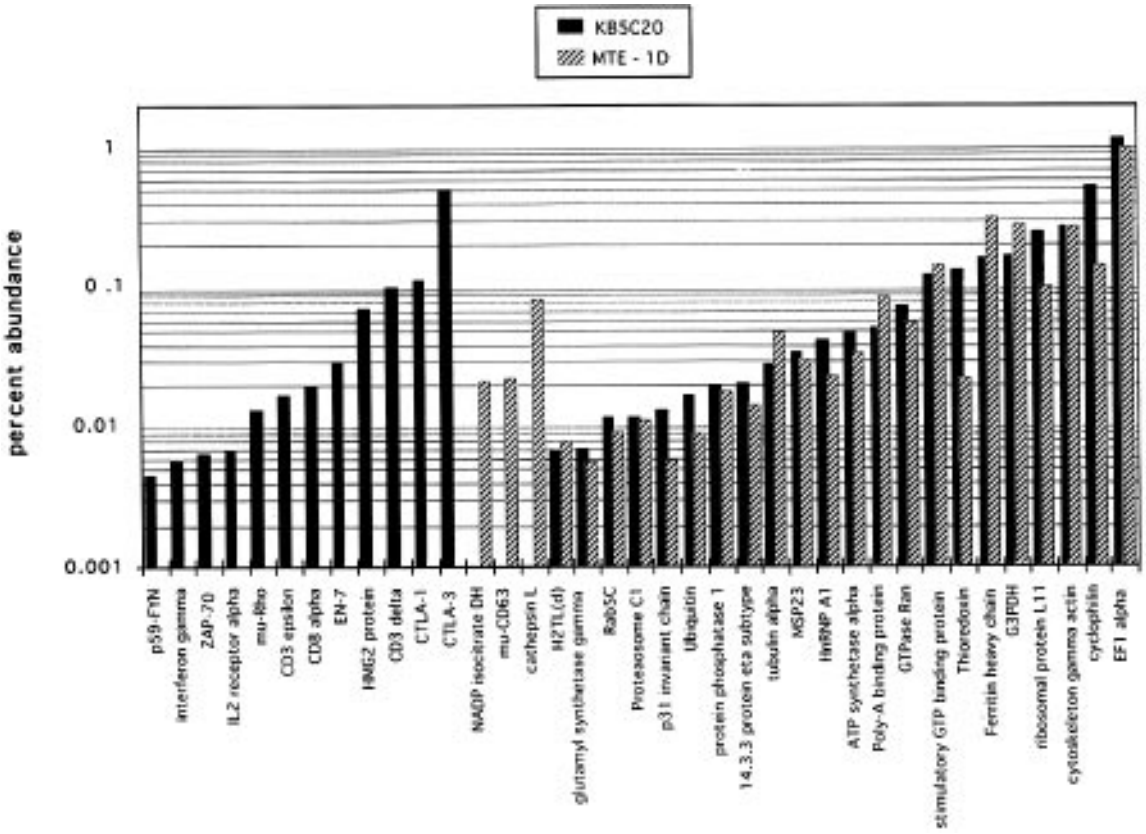


**Figure 4.** Comparison of expression profiles in an epithelial cell line, MTE-1D, and a quiescent T cell clone, KB5.C20. Signals observed on the cDNA clones shown: left, 12 lymphoid-specific genes (black); middle, three epithelial-specific genes (grey); right, ubiquitous genes. The averaged corrected intensity is ordered by increasing values on a logarithmic scale relative to γ actin (abundance 0.3%).

the concentration of the hybridizing sequences in the complex probe (10,17). To control variability resulting from the different amounts of DNA bound to the filter due to spotting, growth of bacteria and DNA binding efficiency we performed the following experiments.

After hybridization with a complex probe prepared from the MTE-1D cell line, each clone giving a signal on the filter was quantified. The intensity obtained for the first colony of a quadruplicate (see above) is plotted against that obtained with the fourth. The diagram (Fig. 2A) shows dispersion around the diagonal resulting essentially from variations in the amount of DNA in each spot. The same filter was hybridized with a vector probe and the signals quantified. The intensity obtained with the complex probe was divided by that measured with the vector probe. The resulting plot, displayed in Figure 2B, shows a marked reduction in the dispersion.

The last diagram (Fig. 2C) shows the reproducibility between two independent hybridizations of the same filter with complex probes made from the same batch of total RNA. The mean of the four signals (after vector correction) for all clones from one hybridization is plotted against that from the other hybridization. The reproducibility obtained is satisfactory enough to perform subsequent analysis on the means of reproducible values obtained from two independent hybridizations. The 26 genes shown in Figure 1C satisfy the criteria and further analysis can be performed on these hybridizations. The point lying off the diagonal corresponds to c554, whose *in vitro* transcribed RNA has been added to the two complex probes in different amounts. As expected, the ratio in the two experiments is close to 10 (see Fig. 2).

## Comparison of sensitivity threshold between complex probe hybridizations and Northern blots

To compare the sensitivity of hybridization with complex probes with that of Northern blots, two reference genes were selected after hybridization with MTE-1D RNA (Fig. 1C): the p31 invariant chain (estimated abundance 0.0057%, close to the threshold) and Rab 5c (estimated abundance 0.0094%). Labeled probes corresponding to these two cDNA clones were successively hybridized with a Northern blot obtained from a gel loaded with serial dilutions of MTE-1D RNA (25–0.25 µg). As shown in Figure 3, the hybridization signals with p31 and rab5c cDNA are detected in lanes where 6.5 and 5.2 µg RNA respectively were loaded. The signals obtained with these two reference clones are close to and twice the threshold defined with the complex probes (made from 25 µg total RNA) respectively. Thus the threshold on Northern blots is reached with approximately four times less total RNA. In conclusion, the sensitivity of Northern hybridization seems ~4-fold better than that of complex probe hybridization.

## Differential gene expression between two distinct cell types

We used this system to compare mRNA levels in two different cell types, a cytotoxic T cell clone, KB5.C20 (unstimulated), and

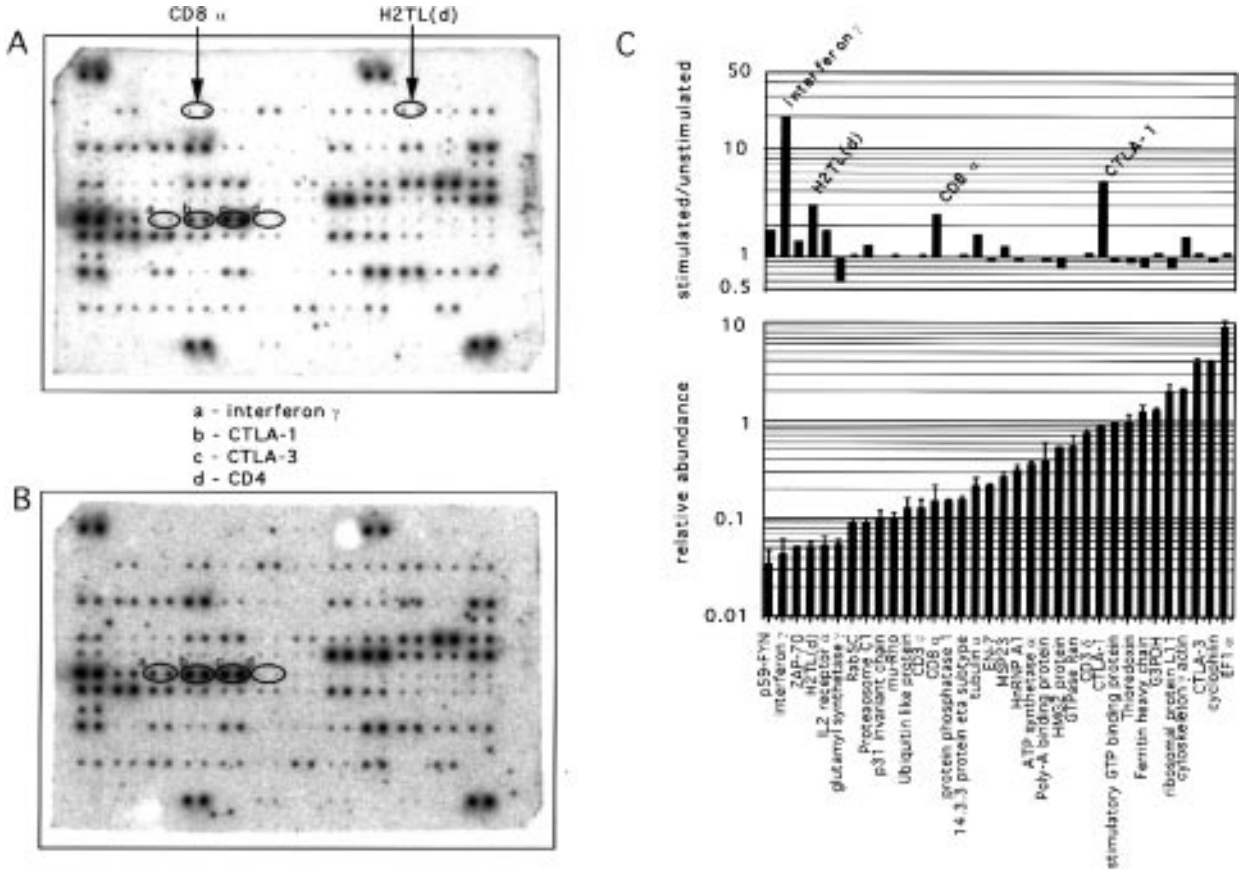


**Figure 5.** Hybridization of an MMA colony filter with complex probes from a cell in two different states. (**A**) Probe prepared from total RNA of a resting cytotoxic T cell line, KB5.C20. (**B**) Probe prepared from the same T cell line after 3 h stimulation by an anti-CD3 antibody. The diagram (**C**) shows at the bottom the intensities for successfully quantified colonies under unstimulated conditions, at the top the variation in expression after stimulation (ratio stimulated/unstimulated).

an epithelial cell line, MTE-1D. Among the 47 clones spotted on the filter, 39 gave a signal with complex probes made with either MTE-1D or KB5.C20 RNA. As shown in Figure 4, 12 genes expressed in the cytotoxic T cell clone are not detected in this epithelial cell line, whereas only three genes are exclusively detected in the epithelial cell. Expression of the other genes is comparable in both cell types, with the exception of the 5-fold higher representation of thioredoxin mRNA in KB5.C20 cells. This expression profile was expected, since candidate genes were chosen to analyze their variations in T cells. Indeed, interferon γ, components of the T cell receptor complex, such as CD3ε and CD3δ, associated molecules, such as CD8α, ZAP-70 and $p59^{fyn}$, and activation markers, such as the IL-2Rα receptor, CTLA-1 and CTLA-3, are expected to be transcribed in a T cell and not in an epithelial cell. Some HMG2 (high mobility group 2) family genes, members of the HMG transcription factor group, are known to be highly expressed in lymphoid cells (21,22). A new member of the small G protein family, mu-Rho (whose sequence is homologous to canine and human rho), was identified with consistent expression in this T cell line as EN-7 small G protein, in agreement with the literature (23). On the other hand, among genes detected only by hybridization with the MTE-1D probe we found cathepsin L, which has been described as expressed in epithelial cells (24), and mu-CD63, described as expressed in kidney and in macrophages after activation (25). Finally, the preferential expression in epithelium of (nuclear encoded) NADP isocitrate dehydrogenase is probably related to the high mitochondria content of epithelial cells in comparison with T cells (26).

## Investigation of differential gene expression upon activation of a cytotoxic T cell clone (KB5.C20)

To analyze transcriptional events occurring upon activation of KB5.C20 cells total RNA was prepared from T cells either in the resting stage or activated by an anti-CD3 antibody.

The same MMA filter was then hybridized with each complex probe, containing the same amount of RNA transcribed from *A.thaliana* cytochrome c554 (Fig. 5A and B; see above). Our positive control (CD8α) is expressed in the quiescent T cell clone (Fig. 5A), while a negative control, CD4, was not found expressed in either unstimulated or stimulated T cells (Fig. 5A and B; 27).

A graphical representation of the results obtained with the RNA from resting T cells is shown in Figure 5C (lower graph). All detected cDNAs were ordered according to the relative abundance of RNA in resting T cells. The ratio between hybridization signals from the same cDNA obtained using probes from stimulated or unstimulated T cells is plotted (stimulated/unstimulated) in Figure 5C (upper graph). Interferon γ (IFNγ) shows a stimulation ratio of ~20. This result is in agreement with previous reports showing IFNγ induction both at the mRNA and at the protein levels after CD3-mediated triggering of the KB5.C20 T cell clone (28). Expression of a number of genes increased with a stimulation ratio

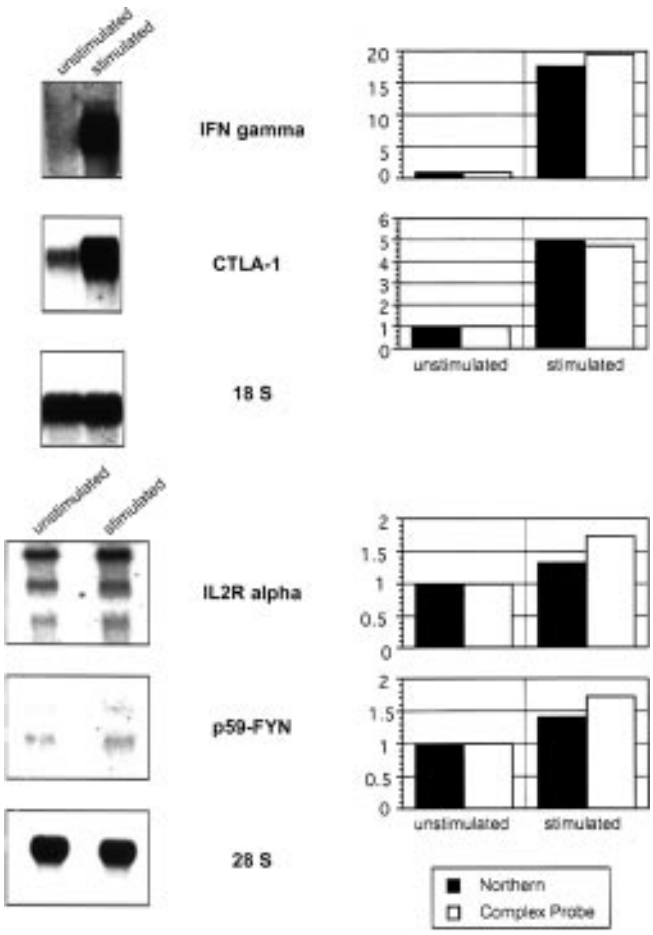


**Figure 6.** Comparison of Northern blotting and differential hybridization for four representative clones. (Left) Northern hybridizations (total RNA from a cytotoxic T cell line KB5.C20, unstimulated and after stimulation by an anti-CD3 antibody) using, from top to bottom, two clones corresponding to highly differentially expressed genes, IFN-γ and CTLA-1, and two corresponding to weak induction, IL2R-α and p59-FYN. The control hybridization with 28S or 18S oligonucleotides used to normalize the data is shown at the bottom of each Northern blot. RNA samples were loaded in the order indicated on the right. (Right) Comparison of figures obtained from quantification of the Northern blot and from the hybridization signature of the same clones on MMA filters. The intensity is represented relative to the signal observed in the unstimulated condition.

of 2 for IL-2Rα and CD8α, while H2TL(d) and CTLA-1 increased with stimulation ratios of 3 and 5 respectively (Fig. 5C, upper graph). The fact that other cytokine encoding genes, such as IL-2 and IL-4, were not induced in response to anti-CD3-mediated activation is characteristic of this type of $CD8^+$ cytotoxic T lymphocyte (CTL) clone (29).

Interestingly, we observed a difference in the behavior of the two serine esterase genes CTLA-1 (granzyme B) and CTLA-3 (granzyme A). CTLA-3 mRNA, quite abundant in unstimulated T cells, did not appear to be increased 3 h after stimulation by an anti-CD3 antibody, whereas CTLA-1 mRNA present at an intermediate level before stimulation was increased 5-fold. CTL grown in the presence of IL-2 are thought to constitutively express their granule content, which includes CTLA-1 and CTLA-3 (30).

In this selected set of genes none seems to be repressed significantly after activation, except possibly for glutamyl synthetase, whose variation is, however, marginal (Fig. 5C). The observed variations are consistent with those measured on Northern blots. Four different genes were tested by Northern hybridization and compared with results obtained with the complex probe (Fig. 6). Northern and complex probe hybridizations appeared to have similar reliability for the detection of strong (CTLA-1 and IFNγ) or weak inductions (IL2-Rα and $p59^{fyn}$).

## DISCUSSION

Reliable means of quickly assessing expression profiles for sets of cDNA clones representing hundreds or thousands of genes are needed to provide this essential complementary information that represents a first step toward functional analysis. Most primary gene activation in eucaryotes requires only ~15–20 min from the initial stimulus to the appearance of mRNA, indicating that if one regulatory gene were to simply activate the next regulatory gene plus a group of functional genes, there would be hundreds if not thousands of such steps in a process as complex as activation or differentiation, lasting many days (31)

Current methods used to determine multiple expression profiles use widely different approaches. Systematic sequencing of a set of randomly chosen clones from carefully constructed cDNA libraries, as implemented by Okubo and co-workers (3), provides frequency data that translates into expression information, but its sensitivity is limited unless very large numbers of clones are analyzed. Rapid PCR-based methods use variations on the original differential display technique (32) to target it to a set of pre-determined sequences (33). While very sensitive, this approach is not quantitative and does not lend itself readily to simultaneous assay of many diverse sequences. The SAGE method (34), in contrast, does provide quantitative information while minimizing the amount of sequencing work through the ingenious use of short, concatenated sequence tags, however, detection and quantification of transcripts present at low levels still requires the analysis of very large numbers of tags.

Hybridization signature methods are inherently parallel and can provide simultaneous expression information on many genes; their sensitivity can be enhanced by modern detection methods and, possibly, by the use of linearly amplified probes. Schena *et al.* (11) have demonstrated such a system using PCR products of *A.thaliana* cDNAs printed in microarrays on glass microscope slides; fluorescence-based detection allows simultaneous two color hybridization, which minimizes the experimental variations inherent in the comparison of independent hybridizations. Thissophisticated technology requires specially developed, state of the art instrumentation for both spotting of the DNA targets and detection of the hybridization signals.

Our MMA system, based on commercially available equipment, can be implemented in any laboratory and is quite generally applicable. We had previously established hybridization signature measurement on high density filters (10), shown quantitative correlation between the signals and the amounts of target and probe DNA and eliminated major artefacts. In the present implementation, designed for more precise expression measurement on a smaller number of clones, the precision has been increased by spotting clones in quadruplicate and the sensitivity reaches 1 in 20 000 using 25 µg total RNA (corresponding to ~0.5 µg mRNA), to be compared with 1 in 50 000 with 2 µg mRNA in the fluorescent system. An external standard allows normalization of signals from independent hybridizations and makes possible precise comparison of different

experiments. The preparation of the complex probe from total RNA makes it possible to use relatively low numbers of cells ($5 \times 10^6$) or small amounts of tissues. Expression levels for a set of 100 genes are obtained in one step using 10–20 times less material than needed for the Northern blot hybridization technique, which would also require much longer. We observed that Northern blot hybridization seems to be four times more sensitive than complex probe hybridization, however, the same reliability in detection of variations is conserved, even for weak inductions. The expression data obtained by this method, just as with a Northern blot, provides a global view of the amount of mRNA at one precise moment, resulting from the balance between transcription and degradation. Of course, gene families give rise to difficulties in this system, just as in Northern hybridizations.

On the set of clones used in this study, a difference in the pattern of expression was easily observed between two cell types (MTE-1D and KB5.C20). We observed consistent expression of a new mouse Rho-like gene in T cells. This gene did not appear to be transcribed at a detectable level in MTE-1D cells, while the other small G proteins Rab5c and Ran were detected (35,36; for a review see 37,38). We easily detected the variation of expression between two different states of a given cell (resting versus activated). Here we found that anti-CD3-mediated stimulation, which is efficient at inducing both perforin- and Fas-based cytotoxic activities in clone KB5.C20 (28), led to an increase in CTLA-1 gene expression without affecting CTLA-3 gene expression. It should be noted that the role in cytotoxicity of the latter granule component is not clear, since cytotoxic function does not seem to be affected in mice rendered deficient in CTLA-3 expression (39). In contrast, CTLA-1 appears to be necessary to the lethal hit delivered by CTL (40).

The MMA method is adapted to analysis of the transcriptional level of a relatively large number of genes in a kinetic context. In the near future the majority of genes will be partially or completely sequenced and this method will be useful for cell typing by expression profile in a number of normal or modified contexts, i.e. during development, as well as in neoplastic or drug-treated cells

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