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Nucleic acid hybridization assays are powerful tools for analyzing the transcriptional activity of genes. Messenger RNA (mRNA) has most frequently been detected by the labor-intensive technique of mixed phase hybridization assay (1, 2). The utility of this method is limited by the fact that nucleic acids bound to nitrocellulose filters have unfavorable reassociation kinetics, and that nonspecific binding of probe can interfere with detection of small amounts of specific mRNA (3, 4). It has been difficult to standardize assays from experiment to experiment because the preparation of filters and their performance characteristics are highly variable. A further disadvantage is that standard assays rely on the use of radiolabeled probes that have a short functional half-life and must therefore be regenerated regularly.

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interleukin-1 (IL-1\(^{\beta}\)) and interleukin-2 (IL-2) mRNA in stimulated monocyte and T lymphocyte cell lines.

**EXPERIMENTAL PROCEDURES**

**Probes**

HUM IL-1, a 1.3-kilobase fragment of the human IL-1\(^{\beta}\) gene cloned in pBR322, was obtained from Smith, Kline, and French (5). A 1-kilobase fragment of the murine IL-2 gene, inserted in pUC18, was provided by Dr. Gordon D. Mille, Toronto, Canada (6). pGEM-3 was purchased from Promega Biotec (Madison, WI). The fragments were cleaved from vector sequences with appropriate restriction enzymes, electrophoresed in an agarose gel, and recovered by electroelution with NA-45 DEAE-cellulose membranes (Schleicher & Schuell) according to standard procedures (7). The purified inserts were labeled with biotinylated cDNA probe, and a conventional enzyme immunoassay that uses a monoclonal antibody for DNA-RNA hybrids to detect the specific mRNA-cDNA complexes. The method has comparable sensitivity to \( \text{P}\text{-based methods and yields results that are quantitative and highly reproducible. Furthermore, the test can be performed using unfracti} \) 

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Quantitative Detection of mRNA by Enzyme Immunoassay

RESULTS AND DISCUSSION

Description of the Hybridization Method—The assay format is based on a hybridization reaction in solution between a biotinylated DNA probe and a complementary mRNA target sequence. Optimum hybridization conditions are determined by reactions of positive and negative control samples with various probe concentrations at different incubation temperatures. Previous studies with plasmid DNA and complementary RNA transcripts have shown that the temperature optimum for perfectly matched targets is 78 °C and that probe concentrations in the range of 20–1000 ng/ml give optimal results (17). Biotin-labeled DNA–RNA hybrids are captured on microtiter plates coated with streptavidin or anti-biotin antibody. (Triton X-100 is added to samples before this step to form micelles with the SDS and prevent desorption of protein from the solid phase.) Following removal of unbound nucleic acids by washing, the amount of biotin-labeled duplexes bound to the solid phase is detected by reaction with an enzyme-labeled monoclonal antibody directed at DNA–RNA hybrids. The preparation and binding properties of the monoclonal antibody have been described previously (18-20). Of note, the reactivity of the antibody exhibits little dependence on the base composition of the nucleic acid hybrid, and the antibody has a very low affinity for either single- or double-stranded DNA. Bound DNA–RNA hybrids are measured by the addition of a fluorescent substrate. For concentrations of RNA between 10 and 3000 pg/ml, the amount of fluorescence generated by the enzymatic reaction is proportional to the amount of captured hybrid. Signals are dependent on probe length, but DNA–RNA hybrids as short as 25 base pairs can still be recognized by the monoclonal antibody.

Comparison of Solution Hybridization/Enzyme Immunoassay Method to Isotopic Slot Blot for Detection of mRNA—The performance of the method for detection of eukaryotic mRNA was first evaluated in an assay for IL-1β mRNA in LPS-stimulated 1H1P-1 cells. The solution phase nonisotopic method was compared with a standard slot blot method (21) using a 32P-labeled probe (specific activity, 2 × 106 cpm/ng). 1H1P-1 cells were passaged for 24 h to provide a stable baseline and then stimulated for 2 h with LPS. Total RNA was extracted by the rapid guanidinium method. One hundred μg of extracted RNA was treated with 23 units of RNase-free DNase I for 15 min at room temperature and then boiled for 3 min. A titration curve of the extracted RNA was prepared in DEPC-treated water and subjected to analysis by either solution phase hybridization followed by enzyme immunoadassay (EIA) detection, or slot blot assay with autoradiogram (Fig. 1). The nonisotopic assay could detect an IL-1 message in as little as 50 ng of total RNA with a signal of 16 ± 6 fluorescence units (fu). The background reactivity generated by the probe was 3 ± 1 fu. The standard filter assay with an isotopic probe and overnight autoradiogram had a comparable detection limit.

Specificity of the Assay for DNA–RNA Hybrids—To prove that the EIA was detecting DNA–RNA hybrids, a separate experiment was performed with human peripheral blood adherent cells as the source of IL-1 mRNA. Total cellular RNA, before and after treatment with 111 RNase, was hybridized with the biotin-labeled IL-1β probe, and the reaction products were tested by EIA (Table I). A total of 4 μg of RNA, extracted by the rapid guanidinium method, was incubated with 1000 units of T1 RNase for 45 min at 37 °C. The nucleic acids were boiled for 10 min and tested in the assay for IL-1 mRNA. Stimulated cells gave a reactivity of 2,180 ± 22 fu which, after digestion with T1 RNase, was reduced to 51 ± 6 fu. Controls to demonstrate the specificity of the assay in-
Quantitative Detection of mRNA by Enzyme Immunoassay

THP-1 cells (2 × 10⁶) were stimulated with LPS (10 μg/ml) and then incubated at 37°C. At indicated times after stimulation, cells were harvested by trypsinization and washed with PBS. RNA was extracted by each of three methods: (a) purification by guanidinium isothiocyanate/cesium chloride centrifugation (Guan/CsCl); (b) extraction by the one-step acid guanidinium isothiocyanate/phenol-chloroform method (Guan/Phe); or (c) recovery of cytoplasmic RNA by a single-step method (cytosol). In all cases, the RNA equivalent of 1.0 × 10⁶ cells was mixed with biotinylated IL-1β probe and hybridized in solution. DNA-RNA hybrids were detected by EIA. Data are expressed as fluorescent units (mean ± S.D.). They represent the values obtained in three different experiments, each performed in triplicate.

| Time (min) | Guan/CsCl | Guan/Phe | Cytosol |
|-----------|-----------|----------|---------|
| 0         | 118 ± 11  | 104 ± 30 | 306 ± 49 |
| 30        | 232 ± 20  | 292 ± 82 | 490 ± 27 |
| 60        | 1,025 ± 76| 548 ± 34 | 1,101 ± 281 |
| 120       | 1,695 ± 214| 830 ± 192| 3,436 ± 228 |

**Fig. 2. Comparison of RNA extraction methods for detection of IL-1β mRNA in THP-1 cells.** THP-1 cells (2 × 10⁶) were stimulated with LPS (10 μg/ml) and harvested 30, 60, or 120 min later. RNA was extracted by each of three methods: (a) purification by guanidinium isothiocyanate/cesium chloride centrifugation (○); (b) extraction by the one-step acid guanidinium isothiocyanate/phenol-chloroform method (■); or (c) recovery of cytoplasmic RNA by a single-step method (□). In all cases, the RNA equivalent of 1.0 × 10⁶ cells was mixed with biotinylated IL-1β probe and hybridized in solution. DNA-RNA hybrids were detected by EIA. Data were normalized to the baseline fluorescent signal at t = 0 min.

**TABLE I**
Specificity of the enzyme immunoassay for detection of DNA-RNA hybrids

| RNA source      | Treatment | Probe   | Fluorescent units (± S.D.) |
|-----------------|-----------|---------|--------------------------|
| Stimulated cells| IL-1β     | 2,180 ± 22 |
| Stimulated cells| T1-RNase  | IL-1β   | 51 ± 6                   |
| Stimulated cells| pGEM      | IL-1β   | 38 ± 6                   |
| Unstimulated cells| IL-1β   | 43 ± 1   |
| Unstimulated cells| T1-RNase| IL-1β   | 32 ± 4                   |
| E. coli tRNA    | IL-1β     | 27 ± 3   |

**Fig. 1. Comparison of solution hybridization/enzyme immunoassay and 3P-based slot blot assay for detection of IL-1β mRNA.** Total cellular RNA was extracted from LPS-stimulated THP-1 by the rapid guanidinium method. One hundred μg of extracted RNA was treated with 23 units of RNase-free DNase I for 15 min at room temperature and then boiled for 3 min. Two-fold dilutions were prepared in DEPC-treated water with 0.5% SDS. The presence of IL-1β mRNA was detected either by solution hybridization and enzyme immunoassay or slot blot assay with 3P-labeled probe. Data for the solution hybridization method are expressed as the mean fluorescence ± S.D. for three replicates. (Background reactivity generated by probe in the absence of RNA samples was 3 ± 1 fluorescent units.)

**TABLE II**
Comparison of RNA extraction methods

| Time (min) | Guan/CsCl | Guan/Phe | Cytosol |
|-----------|-----------|----------|---------|
| 0         | 118 ± 11  | 104 ± 30 | 306 ± 49 |
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Quantitative Detection of mRNA by Enzyme Immunoassay

A novel nucleic acid detection technique is described for the measurement of eukaryotic messenger RNA in biological samples. The procedure involves two steps: a hybridization reaction in solution with a biotinylated DNA probe, and a conventional enzyme immunoassay to detect specific, labeled DNA-RNA hybrids. The method is practical and yields results that are highly reproducible and objective. The nonisotopic detection method avoids the biohazards, inconvenience, and cost associated with use of radioactivity while achieving a sensitivity comparable to $^{32}$P-based methods. It should be noted that although a fluorogenic substrate was used in these experiments, equal sensitivity can be achieved with an alkaline phosphatase-labeled antibody and a colorimetric substrate (22). A second major advantage of the assay over techniques that use autoradiographic detection is the ability to quantitate results easily and accurately. The assays for IL-1$\beta$ and IL-2 mRNA were generally linear over two log$_{10}$ dilutions, and samples containing equivalent amounts of mRNA gave similar fluorescence values. Another advantage of this method is that samples can be tested without the need to extract RNA with organic solvents. However, when maximum sensitivity is required, extracted samples may be required to reduce background reactivity of negative controls.

The methods described in this report can in principle be applied to measure the expression of any gene whose sequence is known. The minimal requirement for the assay is a sequence of 25-50 bases although a probe of 250-350 bases is preferred for maximal sensitivity. When used in combination with a rapid method for recovery of cytoplasmic RNA, the solution hybridization/enzyme immunoassay technique offers a rapid and quantitative means of studying changes in cellular mRNA levels.

Acknowledgments—We thank John R. Jayman and Lisa Goldberg for technical assistance and Donna Dieterich for typing.

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Fig. 3. Titration of extracted RNA from EL4 cells, assayed for IL-2 mRNA by the solution hybridization/enzyme immunoassay method. EL4 cells (2 x 10$^6$) were cultured for 4 h in the presence of phorbol 12-myristate 13-acetate and IL-1, and cytoplasmic RNA was recovered by the single-step isolation method. The RNA extract (solution A, ---), 2-fold (solution A/2, Ci), and 4-fold dilutions (solution A/4, ---) were prepared. Each solution was then serially diluted and assayed for IL-2 mRNA. Results represent mean ± S.D. for three replicate samples.

prepared in DEPC/water with SDS, and then each sample was hybridized with biotinylated IL-2 cDNA at a final concentration of 12.5 ng/ml. Labeled hybrids were detected by EIA (Fig. 3). The peak signal in stimulated cells (2 x 10$^6$) was 939 ± 87 fu, compared with 27 ± 1 fu from an equal number of unstimulated cells and 3 ± 1 fu from probe reacted with buffer alone. Standard deviations for mean fluorescent signals of three replicates were 5 ± 2.6% of the mean. The assay was linear over 2 log$_{10}$ dilutions. Semiquantitative results, obtained by direct inspection of the graph, demonstrate that each of the dilution curves is parallel to the others and shifted by a factor of approximately 2.

Quantitative results were obtained by analyzing the fluorescence data for undiluted mRNA, 1:2 mRNA, and 1:4 mRNA by the parallel line method (16). Six-point calculations were made, selecting fluorescence values between 100 and 500 for each dilution (Fig. 3). This range was selected because the lines appeared to be steepest there and because there was a suggestion of nonlinearity with fluorescence values over 500.

The comparisons made were undiluted to 1:2, undiluted to 1:4, and 1:2 to 1:4. The ratios for these comparisons should have been 2.0, 4.0, and 2.0, respectively. The calculated values were 2.26 with confidence limits of 2.12 and 2.41; 4.77 with confidence limits of 4.44 and 5.12; and 2.08 with confidence limits of 1.94 and 2.22. These values are close to but not identical with the theoretical ones, which confirms the visual impression that concentrated samples give slightly lower fluorescence values than would be expected. The analyses of variance showed no serious deviations from parallelism and no evidence of line curvature. The $g$ values were 0.004, which confirmed assay validity.

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