Use of Flow Cytometry and RT-PCR for Detecting Gene Expression by Single Cells

BioTechniques 21:286-291 (August 1996)

Elissa M. Gaynor, Michael L. Mirsky and Harris A. Lewin
University of Illinois at Urbana-Champaign, Urbana, IL, USA

ABSTRACT

We have developed a method for reliably detecting gene expression by individual, phenotypically defined cells. Cells were sorted by flow cytometry into 96-well plates containing a Nonidet® P-40 (NP40)-based lysis solution. Reverse transcription (RT) of cellular major histocompatibility complex class II DQB and either bovine leukemia virus (BLV) env or tax/rex mRNA was subsequently conducted using gene-specific oligonucleotide primers. Two sequential rounds of PCR were then performed to co-amplify DQB and either BLV env or tax/rex cDNA. The PCR products were electrophoresed in 6% polyacrylamide gels and visualized by ethidium bromide staining. The BLV-infected BL3 cell line was used to establish the sensitivity of the method; cellular and viral mRNA were reproducibly detected in wells into which single BL3* cells were sorted. Additionally, BLV env mRNA from single infected cells was consistently detected in reactions containing as many as 1000 uninfected cells. By using this method, 0.012% ± 0.002% of B cells from a BLV-infected cow with persistent lymphocytosis were found to express BLV tax/rex mRNA, whereas 0.001% expressed BLV env mRNA. The combination of single-cell sorting and RT-PCR provides a powerful new tool to study viral transcription, host responses associated with progression of retroviral infections or other problems requiring determination of the frequency of cells expressing a particular gene(s).*

INTRODUCTION

Methods for the detection and analysis of gene transcription have become increasingly sensitive, thus providing important tools for the study of gene regulation at the cellular level. However, existing methods for detecting mRNA transcripts in single cells require micromanipulation for cell isolation and may not be sensitive enough to permit quantitation of cells containing transcripts of interest in a heterogeneous population (2,6,18). Additionally, existing methods are limited by their inability to identify the cell type(s) expressing the mRNA, by the difficulty in accurately estimating low frequencies of cells expressing a gene and by the restricted number of distinct transcripts that can be detected in a single cell. The use of flow cytometry in conjunction with polymerase chain reaction (PCR) and in situ hybridization has overcome some of these problems (12).

Li et al. (10) introduced the use of fluorescence-activated cell sorting to facilitate the separation of single sperm for PCR-based genetic analysis. We have used a similar strategy to identify and quantitate cells containing the proviral form of the bovine leukemia virus (BLV) as a means for monitoring disease progression (11). In the present study, we incorporated reverse transcription (RT) into our sorting and PCR amplification procedure in order to determine the number of peripheral blood B cells expressing BLV mRNA. This method is shown to be reliable for detecting gene expression by single cells and demonstrates that viral mRNA expression can be accurately detected at cell frequencies of less than 0.015%.

MATERIALS AND METHODS

Cell Lines and Animals

The bovine B lymphoblastoid cell

lines BL3* and BL3° (15) were utilized to determine the sensitivity and reproducibility of a single-cell RT-PCR. BL3° cells are uninfected, whereas BL3* cells are a subline infected productively with BLV.

Peripheral blood was obtained from a BLV-seropositive 10-year-old Holstein-Friesian cow with persistent lymphocytosis (PL). Methods and criteria for diagnosis of BLV infection and PL have been described previously (9). B cells in this cow comprised 69% of the peripheral blood mononuclear cells (PBMC), of which 85% were infected with BLV as determined by using single-cell provirus detection (11).

Flow Cytometry and Cell Lysis

Cultured cells were washed twice in cold phosphate-buffered saline and adjusted to approximately 2×10^6 cells/mL. Aliquots of 1×10^3 cells were dispensed into 0.75-mL tubes (Robbins Scientific, Sunnyvale, CA, USA) and kept on ice until cell sorting. Peripheral blood B lymphocytes were labeled with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine IgG, heavy and light chains-specific (Accurate Chemical, Westbury, NY, USA), as described previously (11). A laser-based flow cytometer equipped with a single cell deposition device (EPICS™ 751 with Autoclone; Coulter Electronics, Miami, FL, USA) and a Cicero™ data acquisition and sorting system (Cytomation, Fort Collins, CO, USA) were used to sort BL3* and BL3° cells on the basis of forward and 90° light scatter. FITC-labeled B lymphocytes were sorted on log green fluorescence. The accuracy of single-cell sorting was checked by sorting cells onto microscope slides and examination by fluorescence microscopy. The desired numbers of cells (1–1000) were sorted into wells of 96-well microplates (MJ Research, Watertown, MA, USA) contain-

Table 1. Primers Used for Reverse Transcription and Amplification of BLV *env*, *tax/rax* and *DQB* cDNA

Primer	Locus	Sequence (5'–3')	Position	Final Primer Concentration (μM)			Fragment Length (bp)
				RT	PCR 1	PCR 2	
1	<i>DQB</i>	AATCCGTCACCGAGCAGA	349–366	0.02	0.008	-	-
2	<i>DQB</i>	CTTCACCAACGGGACGGAG	49–67	-	0.008	0.4	1 + 2 = 317
3	<i>DQB</i>	GGGAGATGGTCACTGTAGGTTC	288–309	-	-	0.4	2 + 3 = 260
4	<i>ENV</i>	TGGGAGACTTGGCACAGAAA	5049–5068	0.4	0.4	-	-
5	<i>ENV</i>	CTATCCGGCAGCGGTCA	288–304	-	0.4	0.4	4 + 5 = 419
6	<i>ENV</i>	GGATGGGCCGACAGAGA	4898–4914	-	-	0.4	5 + 6 = 284
7	<i>TAX</i>	ACATGCAGTCGAGGGAACAA	7450–7469	0.4	0.4	-	-
8	<i>TAX</i>	CTGAAAGCCTTCAAATGCC	4807–4826	-	0.4	0.4	7 + 8 = 286
9	<i>TAX</i>	TGAGCGTGGTGTCTGATCCT	7368–7387	-	-	0.4	8 + 9 = 204

ing 10 μL of lysis solution consisting of 150 mM KCl, 100 mM Tris-HCl, 6.25 mM MgCl₂, 0.05% Nonidet® P-40 (NP40; Sigma Chemical, St. Louis, MO, USA), pH 8.3, and 8 U recombinant RNasin™ (Promega, Madison, WI, USA). The volume of the droplet was approximately 3.6 nL for one sorted cell and 3.6 μL for 1000 cells. For experiments requiring ≥10⁴ BL3° cells per well, cells were manually counted, diluted to 2 μL and distributed directly into the lysis solution.

Primers for RT-PCR

Three sets of oligonucleotide primers were used for RT-PCR (Table 1). Primer sets used for the detection of BLV *env* and *tax/rax* cDNA were designed to cross a splice junction (14,16), ensuring that the products of amplification did not originate from DNA. Primers for detecting exons 2 and 3 of the major histocompatibility complex (MHC) class II *DQB* mRNA cross an intron (19) and were used as a control for cell placement, RT and PCR amplification.

RNA Isolation and Reverse Transcription

Total cellular RNA from BL3* and BL3° cells was isolated using Tri Reagent™ (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. The RNA was used for establishing optimum conditions for RT-PCR and as a positive control in sorting experiments. For

the latter, 0.1 ng of BL3* RNA was added to three wells of each plate immediately after cell lysis. Ten microliters of RT mixture were then added to each well of the plates to provide 250 μM each dNTP (United States Biochemical, Cleveland, OH, USA), 1 mM dithiothreitol (DTT) (Life Technologies, Gaithersburg, MD, USA), 10 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies) and primers (Table 1) in a final volume of 20 μL. Low concentrations of the *DQB* primers were used in the RT and subsequent PCR to minimize nonspecific interference with the amplification of the less abundant BLV mRNA (data not shown). Samples were overlaid with 10 μL of Chill-out™ 14 liquid wax (MJ Research). RT was carried out by incubating the plates at 42°C for 60 min in a 96-well PTC-100™ Thermal Cycler (MJ Research). Reactions were terminated by heating the plate at 94°C for 10 min. Plates were frozen at -70°C or used immediately for PCR amplification.

PCR Amplification of *DQB* and BLV *env* or *tax/rax* cDNA

Cellular and viral cDNA were amplified using two separate rounds of PCR. For the first round of amplification, 30 μL of PCR mixture were added to each sample, providing a final concentration (accounting for concentrations in the RT reaction) of 200 μM each dNTP, 3.05 mM MgCl₂, 30 mM KCl, 20 mM Tris-HCl, pH 8.3, 1 U *Taq* DNA polymerase (Perkin-Elmer, Nor-

walk, CT, USA) and *DQB* and BLV *env* or *tax/rax* primers (Table 1) in a final reaction volume of 50 μL. An additional 10 μL of wax were added to each well immediately before amplification. Cycling parameters for the first round of PCR were 4 min at 94°C; 40 cycles of 94°C for 1 min, 53°C for 2 min, 72°C for 1 min and a final extension step of 5 min at 72°C. For the second round of amplification, 2 μL of the first round PCR product were transferred to two independent mixtures, providing a final concentration (accounting for concentrations in the first round of PCR) of 200 μM each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, either *DQB* or *env* or *tax/rax* primers (Table 1) and 1 U *Taq* DNA polymerase in a volume of 52 μL. Each well was then covered with 20 μL of wax. Second round cycling parameters were 94°C for 4 min; 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 30 s and a final extension step of 5 min at 72°C.

Aliquots of the second round BLV *env* (3–5 μL) or *tax/rax* (10–20 μL) and *DQB* (3–5 μL) products from each well were combined and electrophoresed in 6% polyacrylamide minigels (Hoefer Pharmacia Biotech, San Francisco, CA, USA), stained with ethidium bromide and visualized on a UV transilluminator. To confirm the specificity of the PCR products, the *DQB*, *tax/rax* and *env* products were digested with the restriction enzymes *Hae*III, *Bgl*II, and *Rsa*I (Life Technologies), respectively. Digestion of 20-μL second-round PCR

Research Reports

Table 2. Detection of Viral and Cellular Gene Expression After RT-PCR of a Single BLV-Infected BL3* Cell Among Varying Numbers of Uninfected BL3° Cells

PCR Product	Ratio of Sorted BL3:BL3° Cells per Well						
Detected	1:0	1:1	1:10	1:100	1:1000	1:10000	0:0
<i>DQB</i> ^a	87.5% (210/240)	91.7% (11/12)	100% (36/36)	100% (48/48)	100% (60/60)	100% (12/12)	0.0% (0/58)
BLV <i>env</i> ^b	90.5% (190/210)	90.9% (10/11)	88.9% (32/36)	85.4% (41/48)	85.0% (51/60)	0.0% (0/12)	4.5% (4/88)

^aNumbers in parentheses in row are number of wells with *DQB* product over the number of wells tested.
^bNumbers in parentheses in row are number of wells with BLV *env* product over number of wells with *DQB* product (operational sensitivity).

products was performed by using 10 U of the appropriate restriction endonuclease in a final volume of 25 µL. Restriction fragments were visualized as described above.

Data Analysis

The frequency of B cells containing viral mRNA was estimated using a computer program (SCDPD) developed for single-cell PCR methodology (11). The presence of a cell in each reaction well was confirmed by the presence of the *DQB* product, and the cell was identified as expressing *tax/rex* or

env when these transcripts were detected by RT-PCR. The operational sensitivity, a measure of the efficiency of detection of viral transcripts, was calculated as the proportion of the wells containing *DQB* cDNA that also contained BLV *env* cDNA when testing BL3* cells (11).

RESULTS

Sensitivity of RT-PCR Using Isolated RNA

By using small quantities of total RNA extracted from BLV-infected and

uninfected cell lines, the optimal conditions were established for RT and subsequent co-amplification of viral and cellular cDNA. As little as 10 pg of BL3* RNA provided sufficient template for successful RT-PCR amplification of BLV *env* and *DQB* mRNA (Figure 1). RT-PCR using BL3° RNA yielded only the cellular *DQB* gene product; PCR products were not observed in wells in which RNA or MMLV reverse transcriptase was omitted (Figure 1).

RT-PCR Amplification of BLV *env* and *DQB* from Single BL3* Cells

The efficiency with which both viral and cellular mRNA from an individual cell could be detected simultaneously was determined by sorting single BL3* cells into individual wells of 96-well plates and subjecting them to in situ lysis and RT-PCR. A total of 240 such wells were analyzed (Figure 2, Table 2). Cellular *DQB* cDNA was observed in 87.5% (210/240) of the wells, and 90.5% (190/210) of the *DQB*⁺ wells also contained BLV *env* product. BLV *env* was not observed in the absence of *DQB* in these wells. The *DQB* PCR product was not observed in any of 88 wells with no cells; however, viral *env* product was present in 4.5% (4/88) of these negative controls indicating a low level of background contamination with *env* PCR product. Results of this analysis provided an estimate of the operational sensitivity (90.5%) with which *DQB* and BLV *env* mRNA could be reverse transcribed and co-amplified from single cells. Reactions positive for *DQB* but not BLV *env* may have result-

Figure 1. Simultaneous detection by RT-PCR of BLV *env* and *DQB* expression in the BL3* cell line. Marker (*Msp*I digest of pBR322) (lane 1), 1000 pg RNA (lanes 2 and 3), 100 pg RNA (lanes 4 and 5), 10 pg RNA (lanes 6 and 7), BLV *env* gene product cut with *Rsa*I (225-bp and 59-bp fragments confirm the product as BLV *env*; the band at 284 bp is an incompletely digested PCR product) (lane 8), bovine *DQB* gene product cut with *Hae*III (118-, 90- and 52-bp fragments confirm the product as *DQB*) (lane 9) and no RNA (lane 10). In lanes 2–7, + indicates samples reverse transcribed by MMLV reverse transcriptase; - indicates samples to which no MMLV reverse transcriptase was added.

Research Reports

ed from amplification failure of the viral product, the presence of cells in which the provirus was truly latent or a small population of uninfected cells within the BL3* cell line (11). For analysis, it was assumed that the absence of BLV *env* in wells containing *DQB* was caused by failure to detect viral mRNA, thus making the estimate of operational sensitivity slightly more conservative.

Detection of BLV *env* from a Single BL3* Cell among Multiples of Uninfected BL3° Cells

The sensitivity of detection of individual cells expressing viral mRNA among populations of uninfected cells was determined by sorting single BL3* cells into varying numbers of BL3° cells. As expected for uninfected cells, BLV *env* mRNA was not detected in wells containing up to 10⁴ BL3° cells (data not shown). The BLV *env* sequence from a single infected BL3* cell was reliably detected in wells containing up to 10³ BL3° cells (Figure 3, Table 2). Estimates of the operational sensitivity for detection of viral mRNA from a single infected cell among 10, 10² and 10³ uninfected cells were 88.9% (32/36), 85.4% (41/48) and 85% (51/60), respectively (Table 2).

Detection of Viral mRNA in Peripheral Blood B Lymphocytes

To determine the usefulness of the method for detecting viral mRNA in leukocytes from BLV-infected animals, B cells from a BLV-infected cow with PL were sorted and subjected to the sin-

gle-cell RT-PCR protocol (data not shown). *DQB* mRNA was detected in all wells containing from 1 to 1000 sorted B cells, but never in wells containing no cells. The BLV *env* product was identified in 1/14 wells into which 100 B cells had been sorted, 1/24 wells into which no cells had been sorted and 0/80 wells of 1000 B cells, consistent with previous estimates of low-level (<5%) contamination. Accounting for contamination, the frequency of cells expressing *env* mRNA was certainly less than 0.001%.

Because of reported differences in temporal and/or quantitative expression of the various species of BLV mRNA (3,13), we also examined cells for the presence of the doubly-spliced *tax/rex* message. Neither *tax/rex* nor *DQB* products were detected in 30 control wells to which no cells were added. Of 201 of the 1000-cell wells tested, 200 contained the cellular *DQB* PCR product, and 20 of these wells also contained the BLV *tax/rex* product. From these data, the frequency of circulating B cells containing BLV *tax/rex* transcripts was estimated to be 0.012% ± 0.002%.

DISCUSSION

Flow cytometry permits the rapid separation of phenotypically uniform cells from a heterogeneous population, and PCR provides a sensitive means of identifying a single copy of a gene in 10³ cells (11). We have previously utilized these methodologies to identify a BLV provirus in as few as 0.1% of in-

fecting B cells from BLV-seropositive cows (11). Through the incorporation of a detergent lysis and RT step between cell sorting and PCR amplification, we have now shown that viral and cellular mRNA can be detected simultaneously even when the message is of low abundance, e.g., BLV *tax/rex* in B cells (4,7), and when only one cell expressing viral mRNA is present among 10³ non-expressing cells. Notable features of the protocol include utilization of a low concentration of non-ionic detergent (NP40) in the lysis step, providing the most consistently available template for RT-PCR, and a relatively low concentration of reverse transcriptase to achieve optimal mRNA detection (data not shown).

To establish the single-cell RT-PCR method, we used a cell line productively infected with BLV that expresses abundant viral mRNA relative to the low levels expressed by B cells obtained from BLV-infected cattle (17). Although the lower limit of the sensitivity of this method has not been determined, cells from the productively infected FLK cell line are known to contain at least 40 copies of BLV 35S mRNA (5). A conservative estimate of the sensitivity of our method is that as few as 10 copies of mRNA can be detected in a cell, with sensitivity limited by the efficiency (ca. 40%) of the RT step (1). Once a single cDNA molecule is produced, we are confident in our ability to detect it on the basis of conditions established previously for this purpose (8,11). With such a high level of sensitivity, contamination can be a problem and must be avoided by stringent methods (10). Although all the usual precautions were taken, the source of low-level BLV *env* contami-

Figure 2. Representative results of simultaneous detection of bovine *DQB* and BLV *env* mRNA from individually sorted BL3* cells. *Msp*I digest of pBR322 (lane 1), 11 BL3* cells (lane 2), 1 BL3* cells (lanes 3–8) and no cells (lane 9). Samples were taken from one column of a 96-well plate.

Figure 3. Detection of BLV *env* and cellular *DQB* mRNA from individual infected BL3* cells sorted into varying numbers of uninfected BL3° cells. *Msp*I digest of pBR322 (lane 1). Ratios given in lanes 2–8 are numbers of BL3*:BL3° cells.

nation could not be eliminated in the present work.

By using the single-cell RT-PCR methodology, we hope to elucidate the distribution of transcriptionally active BLV-infected B cells in both peripheral and generative compartments of the immune system and further explore the immunogenetic mechanisms of MHC-mediated resistance to the progression of BLV infection (20). By using additional locus-specific primers for RT, it may be possible to ascertain whether transcription of endogenous genes in infected B cells is altered prior to or in association with disease progression. This method also has great potential for monitoring cellular immune responses to vaccination and for monitoring the efficacy of anti-retroviral therapy through detection of changes in the distribution and/or phenotype of cells expressing viral mRNA and those responsible for mediating immunity.

ACKNOWLEDGMENTS

We thank Mr. Gary Durack and Ms. Karen Magin of the University of Illinois Flow Cytometry Facility for their technical assistance and Jon Beever and Dr. Jerry Lepar for critical reading of the manuscript. This project was supported in part by NIH Grant RO1CA59148 and Grant No. 92-37204-7956 from the United States Dept. of Agriculture, National Research Initiative.

REFERENCES

1. Bouaboula, M., P. Legoux, B. Pességué, B. Delpech, X. Dumont, M. Piechaczyk, P. Casellas and D. Shire. 1992. Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. *J. Biol. Chem.* 267:21830-21838.
2. Furukawa, T., G. Zitnik, K. Leppig, T. Papayannopoulou and G. Starnatoyannopoulos. 1994. Coexpression of γ and β globin mRNA in cells containing a single human β globin locus: results from studies using single-cell reverse transcription polymerase chain reaction. *Blood* 83:1412-1419.
3. Haas, L., T. Divers and J.W. Casey. 1992. Bovine leukemia virus gene expression in vivo. *J. Virol.* 66:6223-6225.
4. Jensen, W.A., J. Rovnak and G.L. Cockerell. 1991. In vivo transcription of the bovine leukemia virus *tax/rex* region in normal and neoplastic lymphocytes of cattle and sheep. *J. Virol.* 65:2484-2490.
5. Kettmann, R., G. Marbaix, Y. Cleuter, D. Portetelle and M. Mammerickx. 1980. Genomic integration of bovine leukemia provirus and lack of viral RNA expression in the target cells of cattle with different responses to BLV infection. *Leukemia Res.* 4:509-519.
6. Kumazaki, T., K. Hamada and Y. Mitsui. 1994. Detection of mRNA expression in a single cell by direct RT-PCR. *BioTechniques* 16:1017-1019.
7. Lagarias, D.M. and K. Radke. 1989. Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections. *J. Virol.* 63:2099-2107.
8. Lewin, H.A., K. Schmitt, R. Hubert, M.J.T. van Eijk and N. Arnheim. 1992. Close linkage between bovine prolactin and BoLA-DRB3 genes: genetic mapping in cattle by single sperm typing. *Genomics* 13:44-48.
9. Lewin, H.A., M.-C. Wu, T.J. Nolan and J.A. Stewart. 1988. Peripheral B lymphocyte percentage as an indicator of subclinical progression of bovine leukemia virus infection. *J. Dairy Sci.* 71:2526-2534.
10. Li, H., X. Cui and N. Arnheim. 1991. Analysis of DNA sequence variation in single cells. *Methods: Comp. Meth. Enzymol.* 2:49-59.
11. Mirsky, M.L., Y. Da and H.A. Lewin. 1993. Detection of bovine leukemia virus proviral DNA in individual cells. *PCR Meth. Appl.* 2:333-340.
12. Patterson, B.K., M. Till, P. Otto, C. Goolsby, M.R. Furtado, L.J. McBride and S.M. Wolinsky. 1993. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. *Science* 260:976-979.
13. Powers, M.A. and K. Radke. 1992. Activation of bovine leukemia virus transcription in lymphocytes from infected sheep: rapid transition through early to late gene expression. *J. Virol.* 66:4769-4777.
14. Rice, N.R., R.M. Stephens, D. Couez, J. Deschamps, R. Kettmann, A. Burny and R.V. Gilden. 1984. The nucleotide sequence of the *env* gene and post-*env* region of bovine leukemia virus. *Virology* 138:82-93.
15. Romano, M.J., J.A. Stewart and H.A. Lewin. 1989. Phenotypic characterization of bovine lymphoblastoid cell lines. *Vet. Immunol. Immunopathol.* 23:293-307.
16. Sagata, N., T. Yasunaga, J. Tsuzuku-Kawamura, K. Ohishi, Y. Ogawa and Y. Ikawa. 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. *Proc. Natl. Acad. Sci. USA* 82:677-681.
17. Teutsch, M.R. and H.A. Lewin. 1996. Aberrant expression of immunoglobulin mRNA in bovine leukemia virus infected cattle. *Vet. Immunol. Immunopathol.* (In press).
18. Tong, J., S. Bendahhou, H. Chen and W.S. Agnew. 1994. A simplified method for single-cell RT-PCR that can detect and distinguish genomic DNA and mRNA transcripts. *Nucleic Acids Res.* 22:3253-3254.
19. Xu, A., C. Park and H.A. Lewin. 1994. Both *DQB* genes are expressed in a *BoLA* haplotype carrying a duplicated *DQ* region. *Immunogenetics* 39:316-321.
20. Xu, A., M.J.T. van Eijk, C. Park and H.A. Lewin. 1993. Polymorphism in *BoLA-DRB3* exon 2 correlates with resistance and susceptibility to persistent lymphocytosis caused by bovine leukemia virus. *J. Immunol.* 151:6977-6985.

Received 17 March 1995; accepted 11 January 1996.

Address correspondence to:

Harris A. Lewin
Laboratory of Immunogenetics
Department of Animal Sciences
University of Illinois at Urbana-Champaign
210 Edward R. Madigan Laboratory
Urbana, IL 61801, USA
Internet: h-lewin@ux1.cso.uiuc.edu