Enrichment of transiently transfected mesangial cells by cell sorting after cotransfection with GFP

RUIHUA CHEN, EDDIE L. GREENE, GEORGIANN COLLINSWORTH, JASJIT S. GREWAL, ODETTE HOUGHTON, HAIQUN ZENG, MARIA GARNOVSKAYA, RICHARD V. PAUL, AND JOHN R. RAYMOND
Department of Medicine, Medical University of South Carolina and the Medical and Research Services, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29425

Chen, Ruihua, Eddie L. Greene, Georgiann Collinsworth, Jasjit S. Grewal, Odette Houghton, Haiqun Zeng, Maria Garnovskaya, Richard V. Paul, and John R. Raymond. Enrichment of transiently transfected mesangial cells by cell sorting after cotransfection with GFP. Am. J. Physiol. 276 (Renal Physiol. 45): F777–F785. 1999.—Early passage mesangial cells, like many other nonimmortalized cultured cells, can be difficult to transfect. We devised a simple method to improve the efficiency of transient protein expression under the transcriptional control of promoters in conventional plasmid vectors in rat mesangial cells. We used a vector encoding modified green fluorescent protein (GFP) and sterile fluorescence-activated cell sorting (FACS) to select a population consisting of >90% GFP-expressing cells from passages of nonimmortalized cultures transfected at much lower efficiency. Only 10% transfection efficiency was noted with a β-galactosidase expression vector alone, but cotransfection with GFP followed by FACS and replating of GFP+ cells yielded greater than fivefold enrichment of cells with detectable β-galactosidase activity. To demonstrate the expression of a properly oriented and processed membrane protein, we cotransfected GFP with a natriuretic peptide clearance receptor (NPR-C) expression vector. Plasmid-dependent cell surface NPR-C density was enhanced by 89% after FACS, though expression remained lower in selected mesangial cells than in the CHO cell line transfected with the same vector. We conclude that cotransfection of rat mesangial cells with GFP, followed by FACS, results in improvement in transient transfection efficiencies to levels that should suffice for many applications.

automated cell sorting; adenovirus; lipofection; liposome

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
targeted animals as the source for primary cell cultures. A fifth method uses microinjection of vectors into cell cytoplasm or the nucleus (7). Although each of those techniques has considerable potential merit, they all suffer from drawbacks that limit their practical use or widespread availability. For example, the generation, characterization, and maintenance of viral stocks for expression constructs or liposomes is labor intensive and may present biosafety containment issues, resulting in requirements in time and resources beyond the reach of many laboratories. Similarly, the generation of stable strains of transgenic mice requires significant time and specialized technical expertise, and the transgenes may or may not be expressed in the tissue of interest. Thus these methods could be highly satisfactory for the intense study of a small number of targeted manipulations, but are less suitable for broad mapping or screening efforts.

We sought to devise an efficient and less resource-intensive method for induction of transient protein expression for use in signal transduction studies in renal mesangial cells, a limited-passage nonimmortalized culture system that is notoriously resistant to transfection. For our studies, we used a cotransfected marker to identify and study only the transfected target cells. This general approach has been useful in other cell lines (4) when increased transfection efficiency was necessary. The advent of green fluorescent protein (GFP) as a biomarker offered the opportunity to simplify the selection of transfected cells. GFP is derived from the jellyfish Aequorea victoria, to which it confers luminescence. Laser-stimulated GFP fluorescence can be detected in intact cells in real time without the need for cofactors, substrates, or other proteins (16). We took advantage of this property to isolate transfected mesangial cells post hoc using sterile fluorescence-activated cell sorting (FACS). We found that cells thus obtained could be replated in either flasks or six-well clusters, and subsequently maintained for at least 72 h. In cells selected for high GFP expression, we report that the transfection efficiency and/or expression levels of two different cotransfected cDNAs were considerably improved over levels seen in nonselected cells.

MATERIALS AND METHODS

Chemicals. Lipofectin, Lipofectamine, cell culture media, serum, and antibiotics were from Life Technologies (Gaithersburg, MD), and culture flasks were from Costar (Cambridge, MA). 125I-labeled atrial natriuretic peptide (ANP) was purchased from DuPont New England Nuclear (Boston, MA). DOPPER {1,3-dioleoyloxy-2-(6-carboxyspermyl)-propyl}amid was from Boehringer Mannheim (Indianapolis, IN). Chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Plasmids. The vector encoding a GFP mutant (phGFP-S165T) was purchased from Clontech (Palo Alto, CA). This vector encodes a mutant GFP that contains >190 silent nucleotide changes to optimize the coding sequence based on human codon-usage preferences (15), and a mutation at residue 165 (Ser→Thr), which results in enhanced fluorescence and a single excitation peak at 490 nm. Native GFP is a 238-amino acid protein (26) with a major absorbance peak at 395 nm (ultraviolet) and a smaller peak at 475 nm (blue light) (6). Because living cells tolerate blue light better than ultraviolet light, the minor peak is better suited for intact cell applications. The red-shifted excitation peak of the modified GFP reduces photobleaching and allows for the use of the 488-nm line on the argon lasers used in many fluorescence microscopes and fluorescence activated cell sorting machines.

The pSV-β-gal expression vector (Promega, Madison, WI) is a general approach has been useful in other cell lines (4) when increased transfection efficiency was necessary. The advent of green fluorescent protein (GFP) as a biomarker offered the opportunity to simplify the selection of transfected cells. GFP is derived from the jellyfish Aequorea victoria, to which it confers luminescence. Laser-stimulated GFP fluorescence can be detected in intact cells in real time without the need for cofactors, substrates, or other proteins (16). We took advantage of this property to isolate transfected mesangial cells post hoc using sterile fluorescence-activated cell sorting (FACS). We found that cells thus obtained could be replated in either flasks or six-well clusters, and subsequently maintained for at least 72 h. In cells selected for high GFP expression, we report that the transfection efficiency and/or expression levels of two different cotransfected cDNAs were considerably improved over levels seen in nonselected cells.

F778 ENHANCED TRANSFECTION OF MESANGIAL CELLS

Isolation and culture of rat mesangial cells. We obtained mesangial cells from Sprague-Dawley rats (150–200 g) using standard sieving techniques, as previously described in detail (23, 24). Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂, and subcultured every 1–2 wk by trypsinization. Cells were plated at a density of 2–5 × 10⁵ cells/ml in RPMI medium (pH 7.3) supplemented with 1 mM HEPES, 5 mg/dl insulin, and 20% FCS. Cells used were from passages 4–15.

Transfection. Nearly confluent cells were exposed to 1 ml of serum- and antibiotic-free RPMI medium containing 10 µl Lipofectamine, Lipofectin, or DOPPER, and 1 µg phGFP-S165T/100 mm² cell culture surface area. In cotransfection experiments, 1 µg of pSV-β-gal or pCDNA3-NPR-C/ml transfection mix was also included. The ratio of 10 µl of lipid per 1 µg of DNA was chosen because this ratio delivers the most efficient expression of plasmid DNA in CHO cells (10), which are commonly used for transient expression studies. After incubation at 37°C for 2 h, the transfection medium was aspirated and replaced with growth medium. Cells were examined by fluorescence microscopy were incubated for 24–48 h. For cell sorting, cell monolayers were washed with PBS containing 0.5% BSA, and then detached from dishes by trypsinization.

Flow cytometry and sterile cell sorting. Cells were pelleted by centrifugation, resuspended in PBS containing 0.5% BSA to a final density of 2.5 × 10⁶ cells/ml, and filtered through a nylon membrane to remove cell aggregates. Flow cytometry and cell sorting for GFP and propidium iodide (PI) fluorescence were performed using a FACSStar Plus (Becton Dickinson, San Jose, CA) with INNOVA 70-4 argon laser tuned to 488 nm. Data acquisition and analysis were performed with CellQuest software. A minimum of 10,000 events was collected for each analysis. GFP signals were detected with a 530/30-nm bandpass filter, and PI signals with a 630/22-nm bandpass filter. To facilitate the recovery of large numbers of transfected cells, we performed presorting triggered by fluorescence; we performed the final sort into GFP+ and GFP− populations using forward scatter as a triggering signal. Where applicable, PI+ cells were excluded on both sorting...
ENHANCED TRANSFECTION OF MESANGIAL CELLS

F779

runs. The resulting cells were resuspended in growth medium and allowed to reattach to culture dishes. Expression of GFP and/or cotransfected proteins was analyzed 18–72 h later.

**RESULTS**

In pilot studies, we determined the optimal concentrations of transfection vehicles and the length of incubation required for the best expression of GFP. Nearly confluent cells seeded in six-well dishes were exposed to three different lipid transfection vehicles (Lipofectin, DOSPER, and Lipofectamine) in varying amounts for varying time periods, with and without serum in the transfection mix, along with 1 μg phGFPS165T expression plasmid. Cells were examined at 24 and 48 h, both for expression of GFP (by fluorescence microscopy) and for gross evidence of toxicity. Only 7% of cells were GFP + with Lipofectin (n = 5). Better expression levels were obtained with DOSPER (27 ± 3%, n = 3) and Lipofectamine (31 ± 5%, n = 5). DOSPER required incubation periods of 8–12 h with mesangial cells, whereas optimal results were obtained with Lipofectamine with only a 2-h incubation in serum-free medium. Longer incubations with Lipofectamine resulted in obvious toxicity (i.e., cell detachment and formation of blebs). DOSPER appeared to be less toxic to mesangial cells. However, we chose Lipofectamine for further study due to the convenience of the brief incubation time.

All of the results reported below were obtained from cells transfected with phGFPS165T using a 2-h incubation with Lipofectamine. After ~36 h, the percentage of GFP + cells was measured. In nine separate experiments, transfection efficiency varied between 10% and 40%, depending, to some extent, on the minimum fluorescence intensity accepted for GFP positivity. These efficiencies are higher than are typically achieved, most likely due to the sensitivity of the GFP assay, and due to our efforts to optimize the transfection conditions. Figure 1 depicts the results of an experiment in which we selected cells for both GFP expression and PI exclusion. Cells that were unable to exclude PI from the nucleus were assumed to be nonviable. GFP +-PI - cells were then allowed to reattach overnight. The next day, the cells were again harvested for flow cytometry. On this second analysis, the cells were >90% GFP + and <1% PI +. These results established that a viable cell population that almost universally expresses the marker protein could be readily obtained and maintained overnight, although expression levels varied considerably from cell to cell. Figure 2 demonstrates the findings on fluorescence microscopic examination of this cell population.

To determine whether selection of GFP + cells could lead to the enrichment of mesangial cells expressing a separate target gene, we carried out cotransfection experiments. We first transfected the pSV-β-gal expression vector together with phGFPS165T. Transfected cells were separated into two different groups based on the intensity of the fluorescent GFP signal. In the experiment shown in Fig. 3, a single round of cell sorting was performed to yield two groups of cells: cells that did not express or had low levels of fluorescence (GFP -) and cells that showed relatively high fluorescence intensity (GFP +). Sorted cells and controls were plated in equal numbers as follows: 1) mock-transfected cells, 2) cotransfected but unsorted cells, 3) GFP - cells, and 4) GFP + cells. For this experiment, PI exclusion was not assessed because it was assumed that nonviable cells could not be replated and, therefore, would not contribute to results obtained after further cell culture. The cells were then stained for β-galactosidase expression 15 h later. Mock-transfected cells had no β-galactosidase staining, and GFP - cells also showed virtually no β-galactosidase staining. A small portion (~10%) of the cotransfected, nonsorted population showed staining for β-galactosidase. In contrast, >50% of cells in the GFP + group stained positively for β-galactosidase, indicating a reasonably good correlation between GFP expression and β-galactosidase expression. Therefore, by selecting cells that expressed high levels of the GFP marker, a greater than fivefold enrichment of target gene (β-galactosidase) expressing cells was achieved.

β-Galactosidase is a widely used marker for transfection efficiency. It is a small and robust protein whose
enzymatic activity can be easily and sensitively detected against the background activity in many mammalian cells. Measurement of the expression of other marker genes could potentially suffer from a lower signal-to-noise ratio. The maintenance of activity of more complex proteins that require extensive posttranslational modifications can be another significant problem. To further establish the usefulness of our approach, we cotransfected cells with GFP and NPR-C. NPR-C was deemed suitable because it is a plasma membrane protein normally expressed by mesangial cells, which requires proper proteolytic cleavage, plasma membrane targeting, orientation, and N-linked glycosylation to bind its ligand. Therefore, ligand binding of transfected NPR-C represents a stringent test of the capability of mesangial cells to process an endogenous protein normally after exposure to the rigors of GFP cotransfection, cell sorting, and replating.

The experimental design, including grouping of the cells, was similar to the experiment shown in Fig. 3; selected cells were above the 90th percentile of GFP fluorescence intensity. The cell surface expression of NPR-C was measured by $^{125}$I-ANP binding, displaceable by excess des(18–22)-cANP-(4–23), a specific NPR-C ligand. Mesangial cells can also express another $^{125}$I-ANP binding site, NPR-A, but ANP is not displaced from this site by the competing ligand (23), and binding to this site would therefore be included in `nonspecific` binding in this assay. The endogenous NPR-C binding (1,800 cpm/10$^6$ cells) was relatively low in these mesangial cells under our culture conditions, which were intentionally designed to suppress expression of endogenous NPR-C (23, 24). The background binding was subtracted to yield the specific NPR-C binding, which resulted from expression of the transfected cDNA, or `heterologous` binding. As shown in Fig. 4, the level of heterologous $^{125}$I-ANP binding was $2,393 \pm 40.4$ cpm/10$^6$ cells in transfected cells before cell sorting. After sorting, the GFP$^+$ cells had $4,533 \pm 1,080$ cpm of $^{125}$I-ANP/10$^6$ cells, an 89% enrichment. In contrast, the level of heterologous $^{125}$I-ANP binding in the GFP$^-$ group was $853 \pm 423$ cpm/10$^6$ cells. Therefore, the upper 10% of cells, in terms of GFP expression,
had more than fivefold higher NPR-C expression than the remainder of the cells, and accounted for ~60% of the total heterologous NPR-C expression in the original unsorted transfected population. To generate a standard of comparison for NPR-C expression, the construct was also transiently transfected into CHO cells. This fibroblast cell line is frequently used for transient transfections because of a well-recognized capacity to efficiently take up and express foreign DNA. Specific binding in this cell line was negligible in mock-transfected cells, and was $13,450 \pm 245$ cpm/10$^6$ cells in cells transfected with the NPR-C construct. Therefore, plasmid-dependent NPR-C density in nonsorted, transfected mesangial cells was only ~18% of the presumably optimal level observed in CHO cells, but this fraction increased to 33% after cell selection.

Fig. 2. A: phase-contrast micrograph of mesangial cells after transfection. B: fluorescence micrograph of same field, showing GFP expression in minority of cells. C: fluorescence micrograph of mesangial cells transfected with GFP and replated after FACS. In contrast to B, virtually all cells fluoresced, although fluorescence intensity visibly varied from cell to cell. D: high-power view of individual GFP-expressing mesangial cell after FACS and replating. The cell exhibits grossly normal morphology and is firmly attached through multiple focal adhesions to underlying substrate.

Fig. 3. Effects of cotransfection with GFP, followed by FACS, on efficiency of expression of β-galactosidase. Cells that express β-galactosidase are stained blue. A: representative phase contrast photomicrographs of mock-transfected cells (top left); unselected cells 24 h posttransfection, with phGFP-S165T and pSV-β-gal vectors (bottom left); cells selected for high level of GFP expression (top right); and cells selected for low or undetectable GFP expression (bottom right). B: counting of percentage of β-galactosidase (+) cells. C: histogram of fluorescence intensity in GFP channel of transfected cells, illustrating windows used for cell separation in this experiment.
enhanced virus of Japan) mon internal liposome method uses the HVJ (hemagglutinating virus of Japan) envelope, which contains HN and F glycoproteins that affect fusion. This method has many potential advantages, including highly efficient transient expression of large DNA packages, but has several disadvantages, including extremely complex preparation requirements and lower vector stability than other methods. More classic transfection methods (calcium-phosphate or DEAE-dextran) can also be toxic to cells, or may result in expression of low copy numbers. In addition, nearly all types of nonreplicating vectors are subject to degradation by nucleases, and can be partitioned into non-nuclear compartments (5). Furthermore, because there is no vertical transfer of the packaged DNA, the number of extrachromosomal copies of DNA can fall dramatically by dilution in replicating cells (2).

In general, viral vectors usually have a higher efficiency than nonviral methods. However, because many separate steps are typically required for the expression of the packaged nucleic acid construct, viral transfection or infection strategies can be limited in several regards. The retroviruses serve as excellent examples of the complexities involved in this process. Retroviruses are RNA viruses that bind to specific cellular receptors that are required for viral docking and subsequent transfer of the viral RNA into the cytoplasm of the recipient cells. The RNA is then converted into proviral DNA by reverse transcription. The proviral DNA must then be transported into the nucleus, where it is converted into double-stranded DNA, which must then randomly incorporate into the genome of the host cell. Therefore, difficulties could arise at multiple steps in this process, including 1) the receptor-binding step, 2) the cytoplasmic-delivery step, 3) the reverse-transcription step, 4) the nuclear-transport step, 5) the step in which single-stranded DNA is converted into double-stranded DNA, or 6) integration into the host cell genome. Thus it is not surprising that retroviruses usually yield low copy numbers, with only one or two integrated copies per cell (11). Moreover, retroviruses can only be expressed in replicating cells.

Other viruses, such as the adenovirus or adeno-associated virus, can function in both replicating and nonreplicating cells. Adenoviruses are very useful in that large DNA fragments can be efficiently delivered to the host cell at high titres. However, because the typical adenoviral vectors are not integrated into the host genome and do not replicate, they are subject to degradation by nucleases and to dilution in replicating cells. In addition, many adenoviral constructs are cytotoxic or immunogenic, or interact with endogenous cellular signaling components. Thus, because of their complexities, viral vectors can pose a number of difficulties. That is not to say that viral vectors are not powerful and useful tools. Rather, they require a very high level of commitment and expertise that may be beyond the capacity of many laboratories.

The current work demonstrates an alternative method to increase the efficiency of transient transfection of plasmid-based expression vectors into cultured rat mesangial cells. The technique could be easily adapted to other cell types.2 Although access to a FACS

---

2 This method has also proven successful in primary cultures of rat vascular smooth muscle cells (X. Gong, G. Collinsworth, E. Greene, B. Egan, and J. R. Raymond; unpublished observations).
instrument is absolutely required, special transfection methods or viral vectors are not needed. We acknowledge that other potential pitfalls of this method could be possible, including low overall yields of purified cells or potentially high hourly costs involved in obtaining the purified cells by sterile cell sorting. In addition, because the mesangial cells are transiently transfected with nonreplicating DNA that does not integrate into chromosomes, the purified cell populations cannot be expanded. Despite those potential limitations, this approach could currently be used effectively by many laboratories.

The principles of this method are straightforward. With the rapid quantitation of GFP fluorescence in living cells provided by FACS instrumentation, the transfection efficiency and assessment of cell-to-cell variability in expression level were simultaneously obtained in each experiment. We observed significant enrichment of mesangial cells that expressed a cotransfected target cDNA among cells selected for GFP fluorescence. Depending on experimental goals, the use of enriched populations of cells expressing the cDNA of interest should increase the signal-to-noise ratio of target protein assays. We also confirmed proper targeting, processing, and orientation of a membrane protein encoded by an expression vector. Transient transfection resulting in an appropriately processed gene product would enable physiologically relevant functional studies, eliminating the pitfall of possible compensatory changes associated with stable expression of the target cDNA.

There are several advantages to using GFP as a selection marker. Expression of GFP in mammalian cells does not typically cause significant biological consequences. Unlike other biomarkers, GFP does not require other exogenously applied cofactors, and can be easily detected in living cells. It is well suited for double-labeling studies in mesangial cells. For example, because PI has a different emission peak, we could use FACS to eliminate nonviable cells while concurrently enriching for GFP⁺ cells. In addition, the quantitative nature of the fluorescence profiles of the cell populations provided by FACS allows for adjustment of fluorescence intensity limits for cells sorting within each experiment. If obtaining a large number of cells is the primary consideration, the detection threshold for the GFP signal can be set at a low level. On the other hand, a higher threshold fluorescence value can be used if more efficient transfection is required for a particular application. This ability to “fine tune” the selection/sorting criteria distinguishes the technique described in this report from other strategies that require cotransfection protocols followed by a post hoc purification scheme. One example of this type of strategy is the pHOOK system from Invitrogen. In that system, cells are transfected with a cDNA that encodes a fusion protein of the transmembrane domain of the platelet-derived growth factor receptor and an extracellular single chain antibody (scFv) raised against the hapten, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one. After transfection, cells are enriched by incubation with magnetic beads coated with this hapten (3).

The success of our procedure, and other marker methods, for selecting transfected cells depends on the tendency for cells that take up the marker DNA to take up the target DNA as well. Various markers other than GFP have previously been used to track and monitor transfection efficiency; in fact, β-galactosidase is frequently used for this purpose. In the absence of precise cell-to-cell quantitation of expression, however, individual successfully transfected cells have generally been assumed to express the marker and target proteins in constant proportion (30). Our data indicate that this assumption is not entirely valid. The efficiency of target gene expression (β-galactosidase or NPR-C) roughly corresponded with the marker GFP expression (Figs. 3 and 4). However, we observed, after cotransfection with GFP and β-galactosidase, that 29.7% of unsorted cells displayed a fluorescent signal, yet only 10% of the unsorted population stained positive for β-galactosidase. Even after cell sorting, not all of the GFP⁺ cells stained positive for β-galactosidase. Only 50% of them did so. The discrepancy between the fraction of cells that show GFP signal and β-galactosidase staining might be due to variability in the proportional level of expression of the respective gene products among cells and/or differential sensitivity of the methods used to detect the proteins. Although we can comfortably conclude that GFP⁺ cells are much more likely to express the cotransfected target cDNAs than are the unsorted transfectants or the GFP⁻ cells, the correlation between marker and target gene expression appears to be substantially <100%.

This initial description of the application of GFP to select transfectants could easily be improved and broadened. Numerous investigators have previously ligated GFP cDNA on either end of the target cDNA insert in expression vectors to observe the intracellular distribution of their protein of interest. Translation of these constructs results in concatenated proteins in which the GFP is aligned end to end on the carboxyl or amino terminus of the target protein. This strategy has been made practical by the relatively small size of GFP, its rather silent nature in mammalian systems, and the availability of commercial vectors designed for easy in-frame construction and expression of GFP fusion proteins. The approach has been validated in numerous recent studies of intracellular protein trafficking and delivery (8, 19, 28). As long as the function of the protein of interest is not disrupted, FACS could be used to select functional transfectants directly. Alternatively, vectors could be constructed that drive the expression of GFP and the target gene from separate open reading frames within the same DNA molecule. Another possible method to enhance our approach would be to use fluorescent nucleotide “paints”, which have become widely available recently. Any of those methods could presumably result in a near-100% correlation between GFP and target gene expression in individual cells. Furthermore, cotransfection experi-
ments could be envisioned with either technique, using multiple autofluorescent protein derivatives of GFP with emission spectra that do not overlap. Mutant green-, blue-, and gold-emitting versions of GFP have recently been marketed by Packard BioScience (Meriden, CT), Clontech, and Quantum Biotechnologies (Laval, Quebec, Canada).

In summary, we have described a method to obtain enriched populations of transiently transfected cells derived from early passage primary cultures of mesangial cells, a cell type that has previously been noted to be quite resistant to transfection. The required reagents are readily available to most laboratories, although FACS facilities may be less accessible. The efficiency of target protein expression in selected cells remains substantially <100%, but further improvements in the method can easily be envisioned. Even in its present form, the improvement in transfection efficiency obtainable with this method should facilitate applications such as reporter vector assays, or bulk expression of epitope-tagged signaling proteins for biochemical or cell biological assays.

This work was supported by grants from the Department of Veterans Affairs (Merit Award to J. R. Raymond), the National Institutes of Health (Grants DK-52448 and HL-03710, to J. R. Raymond and E. L. Greene), Dialysis Clinics Inc. (to R. V. Paul), the South Carolina Affiliate of the American Heart Association (R. V. Paul and J. S. Greulich), and a laboratory endowment jointly supported by the MUSC Division of Nephrology and Dialysis Clinics Inc. (to J. R. Raymond) and MUSC University Research Foundation Awards (to M. N. Gornovskaya and E. L. Greene).

Address for reprint requests and other correspondence: J. R. Raymond, Rm. 829 Clinical Science Bldg., 171 Ashley Ave., Charleston, SC 29425-2227 (E-mail: raymondj@musc.edu).

Received 22 September 1998; accepted in final form 22 January 1999.

REFERENCES


