A Novel Approach to Analysis of Transcriptional Regulation in Human Cells: Initial Application to Melanocytes and Melanoma Cells

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An assay system for transcriptional profile analysis of cultured eukaryotic cells has been developed to simultaneously handle multiple samples in a rapid, sensitive, and internally controlled manner. The methodology incorporates a microtiter plate assay system, a rapid cell-harvest enzyme-assay technique, and the bacterial reporter genes β-glucuronidase and β-galactosidase. We demonstrate, using β-actin and SV40 (late) transcription promoting sequences, that this technically refined microtiter-triton-lysate (MTL) assay methodology can readily differentiate between the transcriptional states of human melanocytes before and after pharmacologic stimulation and malignantly transformed versus normal cell environments. Differences in the transcriptional environments are revealed by the relative expression of transcription element probes. The transcriptional activity ratio of the β-actin compared to the SV40 late transcription promoting sequences was approximately 1:2 in primary cultured melanocytes, 2:1 in 12-0-tetradecanoyl phorbol-13-acetate (TPA)-treated melanocytes and 1:4 in the Tang melanoma cell line. Because this MTL assay methodology can accommodate a panel of transcription element probes, we anticipate that the resultant transcriptional profiles will prove useful in deciphering the diverse transcriptional changes that occur within normally regulated and malignantly transformed cells. *J Invest Dermatol 96:742–746, 1991*

Control of DNA transcription may be considered the primary cellular regulatory mechanism, as subsequent regulation of a gene product temporally must follow this event [1]. Feedback into the transcription-initiation process is modulated through interactions between nuclear factors and DNA regulatory sequences [2–4]. Normal cellular transcription is controlled through diverse pathways that can be influenced by humoral factors, cellular factors, matrix interactions, local environmental changes, and developmental programs [2–6]. Oncogenic events may also result in an altered transcriptional environment by the deletion, alteration, or overexpression of regulatory pathway factors [7].

The development of eukaryotic transcription expression vectors [8] and the techniques for DNA-mediated gene transfer [9–12] has allowed further analysis of transcriptional control in eukaryotic cellular environments. Modulation of transcription in these expression systems is monitored by assaying the enzymatic activity of a reporter protein whose synthesis depends upon the transcriptional activity of DNA elements inserted upstream of the reporter protein's DNA coding sequence. Two commonly used reporter enzymes are chloramphenicol acetyl-transferase (CAT) [8] and luciferase [13,14]. These assay systems are sensitive, but require expensive substrates, light emission detection equipment or time-consuming thin layer chromatography. Further, the standard transcription analysis techniques are tedious, particularly for multiple sample experiments.

A methodology has been developed to assay multiple transcription probes in a rapid, sensitive, and internally controlled manner. This system permits the monitoring of various transcription-factor complex activities through their transcriptional effects on a panel of transcription probes in different cellular transcriptional environments. We believe that this system will identify transcription profiles of cellular states and allow for the subclassification of transcriptional alterations providing a direct approach to unraveling the complexities of transcriptional factor interplay, including, for example, events relevant to the malignant transformation of normal melanocytes. In the present manuscript we establish the feasibility of this approach.

MATERIALS AND METHODS

Cell Culture Human melanocyte cultures were prepared as previously described [15]. Briefly, human neonatal foreskins were treated with trypsin at 4° overnight, the epidermis removed and dispersed to form a single-cell suspension by vortexing in .02% EDTA in phosphate-buffered saline (PBS). Melanocytes were selectively grown in hormone-supplemented medium [15]. Epidermal cells were initially plated at 1×10^6 per 35-mm dish. The Tang melanoma cell line (gift of Dr. Jeffrey Tatro) was carried in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and passed at confluence. All mature cultures were provided fresh medium 3 times weekly and maintained at 37°C in 5% CO_2.

Manuscript received October 8, 1990, accepted for publication December 6, 1990.

This work was presented in part at the Annual Meeting of the Society for Investigative Dermatology, Washington, D.C., May 1990.

This research was supported by the Agriculture Research Service, NIH grant HD 24538 (BAG), and NIH training grant 6M67753-11 (JMG).

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Abbreviations:
$CaPO_4$: calcium phosphate
CAT: chloramphenicol acetyl-transferase
HA: harvest assay
MTL: microtiter-triton-lysate
ONPG: o-nitrophenol-β-D-galactoside
PBS: phosphate-buffered saline
PNPG: 4-nitrophenol-β-D-glucuronide
TPA: 12-0-tetradecanoyl phorbol-13-acetate

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DNA Transfection The transfected expression vectors pβ-Glu and pSV-Glu were a gift of A. Sands, Baylor College of Medicine, TX. Briefly, the pβ-Glu construct was generated by the insertion of the chicken cytoplasmic β-actin promoter upstream of the β-glucuronidase gene (uida) and the pSV-Glu construct was generated by subcloning the SV-40 promoter in the later orientation upstream of the β-glucuronidase gene. The pRSV-Gal construct was a gift of G. McGregor, Baylor College of Medicine, TX. This construct was generated by the insertion of the Rous sarcoma virus long-terminal repeat upstream of the β-galactosidase gene (lac Z).

Plasmid DNA purified by CsCl gradient was transfected into melanocytes using standard calcium phosphate ($CaPO_4$) precipitate methods as previously described [16]. Briefly, for each 35-mm plate of 5×10^4 cells, 1.25 μg of each of the probe vector and internal control vector were added to 60 μl of 250 mM $CaCl_2$ followed by the addition of 60 μl of buffer containing 280 mM NaCl, 50 mM hepes, and 1.5 mM Na_2PO_4 at pH 7.12. After 20 min, 120 μl of the $CaPO_4$ crystals were added to each melanocyte dish. After 5 h the medium was exchanged for fresh medium. The second set of melanocytes was given a 45-second 15% glycerol (in PBS) shock followed by two consecutive washes before fresh medium was added.

DNA was introduced into the Tang melanoma cell line using Lipofectin (BRL) and standard methodology [11]. For each 35-mm plate of 5×10^4 cells, 1 μg of each of the pilot and internal control expression vectors were diluted in 300 μl and then pooled with 300 μl of DMEM previously mixed with 20 μg of lipofectin. The medium was removed from each plate of cells and 600 μl of the lipofectin solution added. After 5 h, 1.5 ml of FBS-supplemented DMEM was added. The medium was changed at 24 h; the cells were harvested and assayed 48 h later.

Transfected cultures were histochemically stained by using a chromogenic substrate for the internal control β-galactosidase enzyme. The cells were fixed with 4% formaldehyde for 1 min followed by two consecutive PBS washes. The transfected cells expressing the β-galactosidase protein were stained by incubation overnight at 37°C in 0.5 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal, BRL), 40 mM Hepes (pH 7.4), 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 120 mM sodium chloride, and 1.2 mM magnesium chloride [17].

Microtiter Plate Assay β-galactosidase (grade VI) and β-glucuronidase (type VII) were purchased from Sigma Chemical Products. Commercial units of the two enzymes are not directly equivalent because they are defined by the cleavage of different substrates: a nitrophenol substrate for the *Escherichia coli* β-galactosidase and a phenolphathaleen derivative for the *E. coli* β-glucuronidase.

Analysis of transcriptional regulation was performed using the microtiter plate as follows: the top central 40 wells were used for test reporter levels; the bottom 40 wells were used for the respective internal control levels, and the first and last eight rows were used for blanking.

Transfected cells were harvested following two consecutive washes with PBS by adding 250 μl of harvest-assay (HA) buffer (0.1% Triton X-100, 0.1 M sodium phosphate [pH 7.3], 50 mM β-mercaptoethanol, and 1 mM magnesium chloride) to each 35-mm plate. The cell debris was scraped and transferred to a microfuge tube, vortexed, and centrifuged. Aliquots (100 μl) of supernatant were pipeted into paired micotiter plate wells for quantitation of β-glucuronidase and β-galactosidase activities. The reaction was started by the addition of 100 μl of 4 mM 4-nitrophenol-β-D-glucuronide (PNPG, Boehringer Mannheim) to assay for β-glucuronidase or o-nitrophenol-β-D-galactoside (ONPG, Sigma) to assay for β-galactosidase. The change in absorbance at 414 ± 10 nm was monitored on a Bio-Rad Ela microtiter plate reader.

RESULTS

Specificity, Linearity, and Buffer Independence of the Assay System Initially this system was tested in order to determine the specificity of the enzymes for their substrates as well as the linear range over which this enzyme assay could be quantified. β-glucuronidase and β-galactosidase diluted in HA buffer (without triton) were added to microtiter plate wells containing PNPG, the intended β-glucuronidase substrate, or ONPG, the intended β-galactosidase substrate. The microtiter plate containing the reaction mixtures was maintained at room temperature and absorbance measurements were taken at multiple time points over 1 h. The absorbance readings increased linearly throughout the assay period for each of the enzymes and its intended substrate (β-glucuronidase/PNPG and β-galactosidase/ONPG), whereas there was no detectable product for either enzyme in the presence of the inappropriate substrate (β-glucuronidase/ONPG and β-galactosidase/PNPG) (Fig 1). The assay was found to be linear throughout the range of the microtiter plate reader to approximately 2.0 OD units. Thus, assay of this reporter enzyme substrate pair demonstrated virtually complete linearity and specificity. Further, two reporter enzyme activities could be followed in a simple parallel manner on a single microtiter plate.

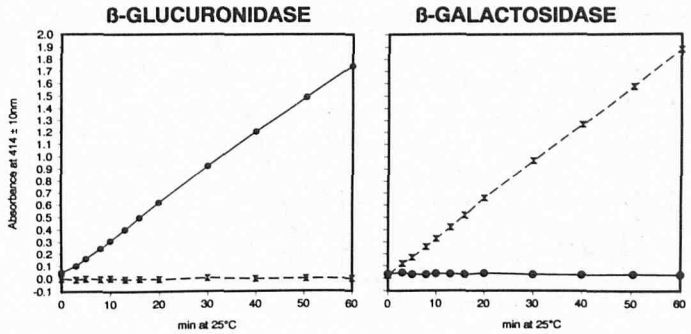

Figure 1. Assay specificity and linearity. β-glucuronidase (200 mU, left panel) and β-galactosidase (5 mU, right panel) were diluted in 100 μl aliquots of HA buffer (without triton) and were added separately to microtiter plate wells containing either 100 μl of 4 mM PNPG *(circles)* or ONPG *(crosses)* in HA buffer (without triton). Absorbance readings at 414 ± 10 nm (y axis) were taken at multiple time points over the 1-h assay period (x axis).

Second, we sought to optimize the cell harvest and enzyme isolation techniques. Standard protocol for obtaining cellular protein for enzymatic assay from transfected cells is a multiple step process: release of cells from the culture dish, centrifugation, buffer change, resuspension, rupture of the cell membranes by repetitive freeze-thaw cycles, and re-centrifugation to obtain a cellular supernatant for enzymatic assay. We developed a single buffer process for both cell harvest and enzyme assay. The key feature of this buffer was the inclusion of the detergent triton X-100, which allowed for the direct isolation of cytoplasmic contents from cells on the culture dish. Using this procedure, the cells lyse directly into the HA buffer as they are scraped from the plate and transferred to a microfuge tube. The supernatant derived after a brief centrifugation of the cell buffer mix can be directly assayed for both β-glucuronidase and β-galactosidase enzymatic activity. This methodology avoids the enzyme loss associated with cell transfer and repetitive freeze-thaw procedures. Further, this system decreases the variability of transcriptional probe and internal control enzyme activities by minimizing the multiple pipeting procedures generally required to test reporter enzymes separately. In the MTL system both the β-glucuronidase and β-galactosidase enzymes are harvested from cells directly into the assay buffer; enzymatic quantitation merely requires pipeting of equal aliquots into microtiter plate wells followed by the addition of the specific chromogenic substrate solutions to initiate the assay. This is in marked contrast to other assay systems such as the traditional CAT assay system, which requires multiple pipeting steps, the use of radionucleotides, thin layer chromatography, autoradiography and further additional assay steps for internal control standardization.

Third, in order to test whether 0.1% triton X-100 would influ-

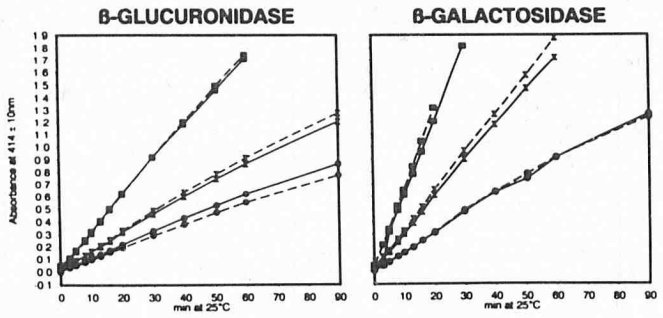

Figure 2. Lack of effect of triton buffer on reporter enzyme activity. *Left,* β-glucuronidase 50 mU *(circles),* 100 mU *(crosses),* and 200 mU *(squares),* and *right,* β-galactosidase 2.5 mU *(circles),* 5 mU *(crosses),* and 10 mU *(squares)* were assayed in the presence of 2 mM PNPG *(left)* or ONPG *(right),* either in the presence *(solid lines)* or absence *(dotted lines)* of Triton buffer. Absorbance at 414 ± 10 nm was read at multiple time points during the 90-min assay period.

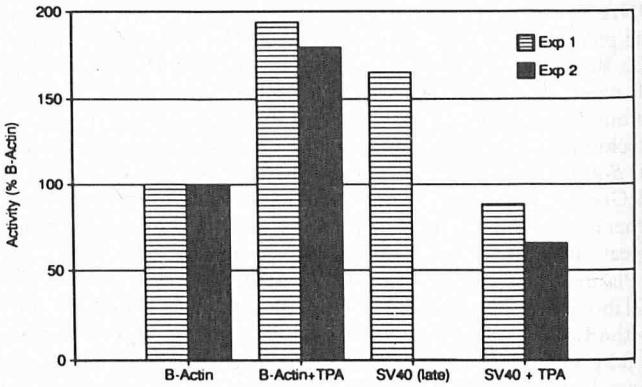

Figure 4. TPA-induced transcriptional modification of human melanocytes. Paired human melanocyte cultures were transfected with the indicated vectors and 24 h later half the cultures were treated with TPA 50 ng/ml. Melanocytes were harvested and assayed for β-glucuronidase and β-galactosidase enzymatic activity 48 h later (72 h post-transfection). Data for two separate experiments are shown, normalized to β-actin expression. Cultures transfected with the SV40 (late) promoter were lost in the second experiment.

ence the enzyme activity of β-glucuronidase and β-galactosidase, titrations of both reporter enzymes were assayed using their respective substrates in the presence or absence of 0.1% triton (Fig 2). Increasing the concentration of the enzymes increased the absorbance readings as expected, but there was no significant effect of triton on the spectrophotometric readings obtained.

Fourth, in order to determine the period of time over which β-glucuronidase and β-galactosidase enzymes were active, β-glucuronidase (10 mU) and β-galactosidase (0.25 mU) were assayed in the presence of both PNPG and ONPG substrates, and activity was followed over a 2-d period (Fig 3). Cross reactivity of the enzyme substrate combinations remained relatively insignificant, although low level hydrolysis of PNPG by β-galactosidase was detected at extended incubation times. β-glucuronidase had relatively linear activity as monitored by PNPG cleavage throughout the 2-d period, but β-galactosidase enzyme activity as monitored by ONPG cleavage reached a plateau at approximately 24 h. Even with this limitation on β-galactosidase activity, overnight assays (less than 15 h) revealed reproducible internally controlled results from as few as 5–10 transfected melanocytes, as determined by histochemical staining of transfected cells in parallel culture. This exquisite sensitivity proved very helpful in light of the low efficiency of transfection achieved (<.1%), particularly with the normal melanocytes and standard transfection protocols.

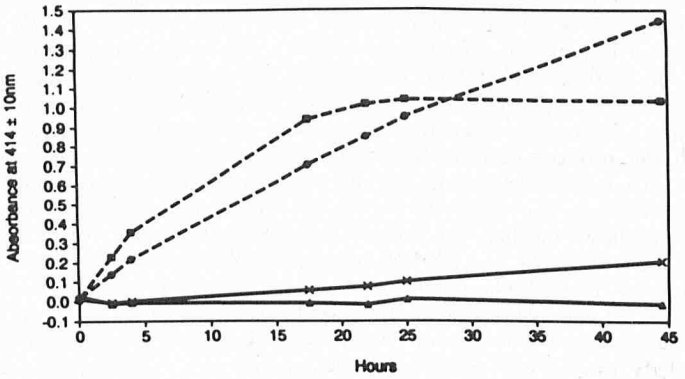

Figure 3. Linearity of enzyme activity over an extended incubation period. β-glucuronidase 10 mU *(circles, triangles)* and β-galactosidase 0.25 mU *(squares, crosses)* were assayed in the presence of 2 mM PNPG *(circles, crosses)* or ONPG *(squares, triangles).* The absorbance of the reaction mixtures was read at multiple time points over a 45-h incubation period. Absorbance readings for β-glucuronidase and its substrate PNPG *(circles)* and β-galactosidase and its substrate ONPG *(squares)* indicate linearity of the reaction for at least 45 and 17 h, respectively.

Influence of TPA on β-Actin and SV40 Transcriptional Probe Activity in Melanocytes In order to test the ability of the MTL assay to detect alterations in cellular transcriptional environments, we compared normal melanocytes in the presence and absence of the phorbol ester TPA. TPA causes marked changes in cellular morphology [18], growth [19], and specific gene expression [20–23], presumably through activation of protein kinase C and the transcription factor complex AP1 [24]. We chose the B-actin and SV40 (late orientation) transcription-promoting regions linked to the β-glucuronidase gene as transcription probes because both were anticipated to exhibit a TPA response. In other cell types, in response to TPA, β-actin levels have been found to both increase and decrease [21,23]; the SV40 promoter has been shown to be activated in the early orientation [25]. We employed the expression vector pRSVGAL, containing Rous sarcoma virus long-terminal repeat (RSV LTR) sequences linked to β-galactosidase, as an internal control, because it drives transcription at a high level in many cell types [26].

Each dish of melanocytes was transfected with 1.25 μg each of the pilot and internal control vectors. Half of the dishes were treated with TPA (50 ng/ml) 24 h after transfection. Melanocytes were harvested and assayed for enzymatic activity 72 h post-transfection (48 h after TPA stimulation). The data were internally controlled against RSV-GAL expression and normalized to β-actin expression. Although transfection efficiencies and thus absolute values for β-glu and β-gal activity varied between the two experiments, the relative effects on the two transcription probes studied were quite similar (Fig 4). Addition of TPA stimulated transcription from the β-actin promoter about twofold and decreased SV40 transcription in the late orientation by about half. Thus, TPA treatment increased the relative expression of the β-actin promoter approximately fourfold compared to the SV40 (late) promoter.

These data demonstrate that this assay system with two transcrip-

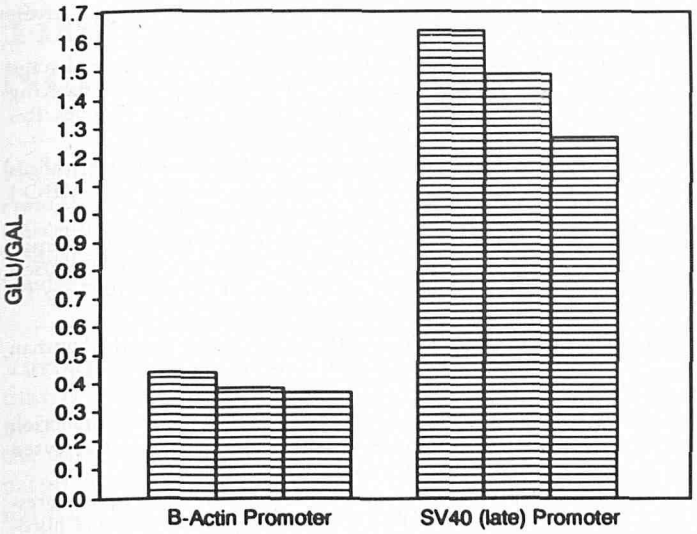

Figure 5. Transcriptional activity of the pilot expression vectors in a human melanoma line. Paired dishes of the Tang melanoma cell line were grown and transfected in triplicate with either the β-actin promoter construct or the SV40 (late) construct. After 48 h, the cells were harvested for the colorimetric triton lysate assay. Absolute values for ratios of the β-glucuronidase to β-galactosidase enzyme activities are shown.

tional promoters as probes can readily detect TPA-induced differences in the transcriptional environment of normal cultured human melanocytes.

Differences in β-Actin and SV40 Transcriptional Probe Activity Between Normal Human Melanocyte and an Established Human Melanoma Line To determine whether the β-actin and SV40 transcription probes could distinguish between the transcriptional environment of melanoma cells versus normal melanocytes, dishes of Tang melanoma cells were transfected in triplicate with the plasmids pB-Glu, pSV-GLU, and pRSVGAL as an internal control. The medium was changed 24 h after transfection and the cells were harvested an additional 48 h later. The internally controlled non-normalized data from this triplicate experiment indicate that the SV40 (late) promoter is drived at a fourfold higher level than is the β-actin transcription promoting region in the Tang melanoma cells (Fig 5). This ratio is twice that observed in normal melanocytes and eightfold that observed in TPA-treated melanocytes. These data demonstrate the potential of this system to profile the transcription differences between transformed and normal cellular environments.

DISCUSSION

We describe an assay system that can be used to probe the transcriptional environment of human melanocytes and to identify changes associated with activation of normal transcription factor pathways or with malignant transformation. Specifically, we have shown that β-actin and SV40 (late) promoters drive transcription at different rates in different molecular environments. In normal human melanocytes, β-actin transcription is approximately half that of SV40. TPA stimulation of melanocytes enhances β-actin transcription and represses SV40 (late) transcription within 48 h, thus altering their relative expression approximately fourfold. This transcriptional profile is vastly different from that in at least one established human melanoma line, in which the SV40 (late) promoter transcription is conversely enhanced twofold and β-actin promoter transcription repressed 50% compared to normal melanocytes.

In the establishment of the MTL assay system, we identified reporter genes whose enzymatic products could be analyzed in a sensitive, rapid, inexpensive, and parallel manner. We chose to employ the uidA gene that encodes the bacterial β-glucuronidase enzyme as our transcriptional reporter gene and the lacZ gene that encodes β-galactosidase enzyme as an internal control for DNA introduction. Both of these genes have been previously used as transcriptional reporters in other assay systems. The β-glucuronidase gene has been primarily applied to plant transcription studies [26,27], whereas the β-galactosidase has been used as a transcriptional reporter, an internal control, and as a histochemical localization marker [17,28–30]. The enzyme assay techniques for both β-glucuronidase and β-galactosidase are well described [27,28]. Colorless substrates are available for each of these enzymes, which yield yellow nitrophenol derivatives upon enzymatic cleavage: 4-nitrophenol-β-D-glucuronide (PNPG) for β-glucuronidase and O-nitrophenol-β-D-galactoside (ONPG) for β-galactosidase. The rate of production of each nitrophenol derivative can be determined by monitoring light absorbance and correlating with enzyme activity using standard curves. In order to maximize simplicity and capacity, we chose to use a microtiter plate reader for taking simultaneous absorbance measurements of the nitrophenol products for the β-glucuronidase and β-galactosidase enzyme assay. The nitrophenol products cleaved from ONPG and PNPG absorb in the blue-violet spectrum. We chose a single filter at 414 ± 10 nm and demonstrated that it could be used to follow the production of both derivatives. We have shown that the bacterial enzymes β-glucuronidase and β-galactosidase are highly specific for their nitrophenol substrates with little cross reactivity and that assay of their activity is essentially linear throughout the range of the microtiter plate reader. In addition, the enzymes are compatible with a single harvest-assay buffer, greatly simplifying the enzyme assay. Further, extended assay incubations with these enzymes are possible, allowing for detection of low levels of enzyme activity and/or for assays of a very small number of cells. Finally, with the first and last row used for blanking, the microtiter plate reader permits 40 internally controlled transfection assays to be performed simultaneously.

The MTL methodology described above has marked advantages over traditional transcription assay techniques. The assay procedure is rapid, inexpensive, and technically simple. Additionally, the system is internally controlled, allowing for standardization of transfection efficiency. Because the assays of the transcriptional probe and internal control enzymes are done in parallel, variability between these measurements is also minimized. Further, the assay is sensitive, requiring as few as 5 to 10 transfected cells with extended overnight assay. Finally, the use of the microtiter plate facilitates rapid data acquisition.

This high-capacity transcription assay methodology could easily be adapted for studying a panel of mutations on a single gene promoter as has been performed using more tedious systems for gene promoters such as β-globin [31]. However, we feel that this system's most promising use is for transcription profile analysis of various cell types. Although the mechanisms by which nuclear factors modulate levels of DNA transcription are still poorly defined, the transcriptional activation or repression in response to changes in these factors can be readily studied through the use of transcriptional probes linked to reporter genes. This approach eliminates other complicating aspects of downstream regulation and focuses strictly upon the nuclear factor modulation of transcription of the selected probe DNA sequence.

One potential application of the methodology is systematic exploration of differences in the transcriptional environment of normal and malignantly transformed cells. We anticipate that such studies will define subsets of different cellular transcriptional environments. The transcriptional DNA probes exhibiting differential expression could be used to isolate and characterize nuclear factors. This methodology may be particularly important in melanoma and

other forms of cancer where it appears that multiple genetic events must occur prior to malignant transformation [32].

In summary, we present a rapid-assay system allowing for internally controlled transcription analysis of multiple samples. We believe that this assay system allows for transcription profile analysis of normal and transformed cellular phenotypes.

We thank Arther Sands for his gift of the β-glucuronidase vectors; Philip R. Gordon, Ph.D, and Mina Yaar, M.D. for thoughtful discussions and melanocyte culture expertise; Richard Maziarz, M.D. for reviewing the manuscript; and Megan Maultsby, Margie Oleksiak, and Tim Rotman for technical assistance.

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