ISOLATION OF TWO UNKNOWN GENES POTENTIALLY INVOLVED IN DIFFERENTIATION OF THE HEMATOPOIETIC PATHWAY, AND STUDIES OF SPERMIDINE/SPERMINE ACETYLTRANSFERASE REGULATION

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ABSTRACT

Differential display identified a number of candidate genes involved with growth and differentiation in the human leukemia cell lines HL-60 and HL-525. Two of these genes were previously unknown, and one is the gene for the enzyme spermidine/spermine acetyltransferase (SSAT). One of our objectives is to isolate and sequence the unknown genes, 631A1 and 510C1, in order to characterize them and determine their functions. The other is to determine how SSAT is regulated, and look at how the polyamines that SSAT regulates effect macrophage differentiation. By screening the CEM T-cell DNA library and the fetal brain library, we were able to identify clones that had inserts with homology to the 631A1 cDNA probe sequence. The insert was amplified using the polymerase chain reaction (PCR) and is currently being sent to the University of Chicago for automated sequencing. The library screens for 510C1 are currently underway, but hybridization of the 510C1 cDNA probe with nylon membranes containing CEM library λ-phage DNA produced strong signal, indicating the gene is there. SSAT experiments identified that the rate-limiting enzyme that marks the polyamines spermidine and spermine for degradation is regulated by PKC-β and a transcription factor called Nrf2. The knowledge of regulation and function of these genes involved in macrophage differentiation will provide new insight into this cellular process, potentially making it possible to discover the roots of the problems that cause cancerous diseases.

INTRODUCTION

Hematopoiesis is the process by which stem cells differentiate into the components of blood in humans. The branch stemming from the myeloid stem cells ends in the formation of monocytes and granulocytes (Figure 1). Final differentiation of a monocyte yields a macrophage. It is possible to study the process of macrophage differentiation in hematopoiesis *in vitro* using a human myeloid leukemia cell line called HL-60, and an HL-60 variant called HL-525, which is differentiation resistant. HL-60 is thought to exist in the phase between CFU-GM and the next step, enabling it to differentiate into neutrophils, or monocytes and macrophages. The fact that HL-525 cells cannot differentiate is the primary difference between the two cell lines. In other words, assuming that the ability to differentiate is at the genomic level, the difference between HL-60 and HL-525 can be attributed to the presence or absence of genes in HL-525, or to altered gene regulation in HL-525.

Another difference between the two cell lines is the presence or absence of the protein kinase C-β (PKC-β) pathway. It has been shown by Tonnetti, Henning-Chubb, Yamanishi, & Huberman (1994) that the PKC pathway is required for macrophage differentiation in HL-60 cells by re-enabling HL-525 to differentiate by transfecting PKC-β deficient HL-525 cells with expression vectors containing cDNA for PKC-β. So if PKC-β is required for macrophage differentiation, then any genes that are dependent on PKC-β for expression might also be involved in differentiation. These genes can be found using a technique called differential display.

First reported by Liang and Pardee (1992), differential display is a powerful technique that highlights the differences in RNA expression in different cell lines. We have used this method to screen for the genes showing the difference in expression between HL-60 and HL-525, as well as between induced and noninduced HL-60. The technique utilizes methods capitalizing on the polymerase chain reaction (PCR). A poly-T plus one base primer that anneals to the 3' poly-A tail of RNA is used along with an arbitrary 5' primer in reverse transcription reaction followed by PCR, making a number of cDNAs for the expressed RNAs in the cell. Sequencing gels are then run to compare the cDNAs of the two cells. Changes in the pattern of the expression are a red flag for genes possibly involved in differentiation. Differences in expression are then confirmed by using the cDNA as a probe on a Northern blot. By using this technique, eight genes were identified for HL-60 or HL-525, both known and unknown. We chose to focus on three of these genes: two unknown genes, temporarily called 631A1 and 510C1, and spermidine/spermine N1-acetyltransferase (SSAT).

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Not that much is known about 631A1 or 510C1. 631A1 is expressed in HL-60 cells, but not in HL-525. It is induced with PMA after 32 hours of incubation, suggesting it somehow plays a role in differentiation (Gavin, 2001). 510C1, on the other hand, is found in HL-525 and not in HL-60. 510C1 has a homeo-domain in the known fragment sequence hinting that it may be a transcription factor of some kind (Gavin, 2001).

Spermidine/spermine N1-acetyltransferase is a rate-limiting enzyme that acetylates spermidine and spermine in polyamine metabolism, tagging them for degradation by polyamine oxidase. Polyamines have long been known to play a role in growth and differentiation, but how these vital cations are regulated is still unclear. It is important to understand the metabolic pathway for polyamines if we are to learn more about polyamine regulation (Figure 2). Ornithine decarboxylase (ODC) is the rate-limiting biosynthetic enzyme, and it was originally thought that polyamines were regulated by the synthetic pathway. However, findings that spermine decreases while spermidine and putrescine increase during phorbol 12-myristate 13-acetate (PMA) induced macrophage differentiation suggests that regulation of polyamines occurs by degradation (Huberman, Weeks, Callaham, & Slaga, 1981). Since SSAT is responsible for the initial steps of polyamine catabolism, how it is regulated is a logical question to ask. We have done a number of experiments with SSAT and polyamines to observe the effects on macrophage differentiation and determine how SSAT is regulated.

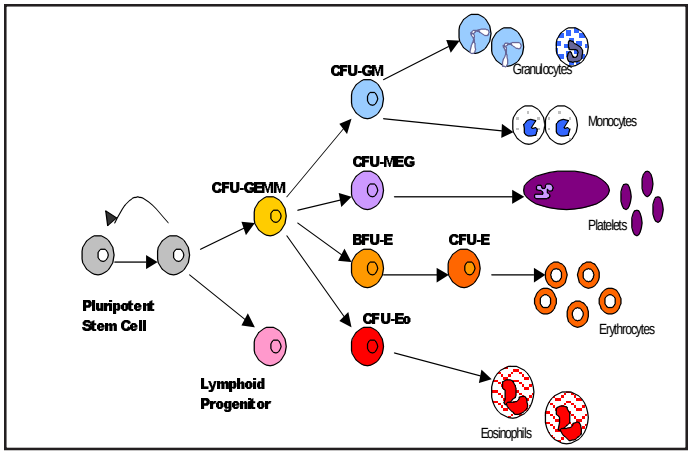

Figure 1. The process of hematopoiesis. HL-60 cells exist in the phase around CFU-GM and can differentiate into granulocytes or neutrophils, and monocytes. Monocytes differentiate further into macrophages. *Figure adapted from Socolovsky, Lodish, & Daley (1998).*

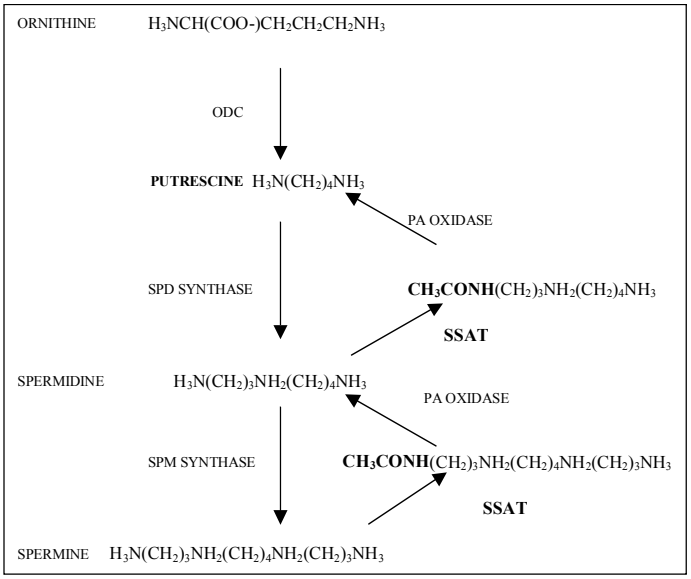

Figure 2. Polyamine biosynthetic pathway. Spermine and spermidine are acetylated by SSAT, marking them for degradation. *Figure adapted from Casero & Pegg (1993).*

MATERIALS & METHODS

631A1

Testing three DNA libraries, a Northern blot determined expression of 631A1 was highest in the human CEM T-cell DNA library from Clontech Laboratories. Polymerase Chain Reaction (PCR) was used to amplify 631A1 cDNA probes. Y1090 strain of Escherichia coli was infected with the CEM library in lambda Zap phage, and plated on 150mm LB-agar $MgSO_4$ plates. We also used human fetal brain plasmid library in pcDNA1, also by Clontech, plated on 150mm LB-agar plus half ampicillin, full tetracycline plates. After overnight incubation at 37 °C, the colonies and plaques were lifted with Magna lift nylon transfer membranes by Osmonics Inc. The CEM membranes were crosslinked with a Stratagene UV Stratalinker-2400 at 1200 µJ. The fetal brain membranes were baked at 80 °C in an oven for one hour. The membranes were then hybridized with P^{32} labeled 631A1 cDNA probe, and exposed to film. The plates were lined up with the developed film. Ten picks were made from the CEM library; five picks were made from the fetal brain library.

The CEM picks were cleaned with 250 µL of phage dilution buffer (35 mM Tris pH 7.5, 10 mM $MgSO_4$, 0.01% gelatin, 0.1 M NaCl, H_2O) and 25 µL chloroform, used to infect Y1090, and plated on 80 mm LB-agar $MgSO_4$ plates. The fetal brain picks were used to streak new 80 mm LB-agar half ampicillin, full tetracycline plates. A second round of lifting and hybridizing was done. This time, six CEM picks were freeze-thawed in water three times. Five fetal brain picks were boiled 10 minutes in colony lysis buffer. The CEM picks were PCR amplified with T7 and T3 primers; the fetal brain picks were PCR amplified with pcF/R primers. Five additional fetal brain picks were made and put in 100 µL LB to be grown up overnight. DNA was prepared with the Qiagen QIAprep Miniprep Kit (250), and then double digested with BamHI and XbaI restriction endonucleases.

The digests and PCR products were electrophoresed on a 1% agarose gel. Two bands from the digest were excised, and two PCR products were subject to a second round of PCR. PCR products were then purified with the Qiagen Qiaquick PCR Purification Kit (50). A fraction of DNA was then digested with EcoRI, and run out on a 1% agarose gel. The vector Bluescript Sk(+) was prepared for subcloning by digesting with EcoRI, EcoRV and BamHI/XbaI. DNA was obtained from gels using the Quigen QIAEX II Gel Extraction Kit.

Ligation reactions were set up for EcoRV digested Sk(+) and blunt ended PCR products, EcoRI digested Sk(+) and EcoRI cut PCR product, and BamHI/XbaI digested Sk(+) and BamHI/XbaI

cut fetal brain plasmid. The ligations were purified by phenol chloroform extraction, and transformed into electrocompetent bacteria by electroporation with a BioRad Gene Pulser (1.8 kV, 20 Ω, 25 μFD). The bacteria were then plated on full ampicillin/tetracycline plates with 100 mM IPTG and X-gal DMFA for blue-white screening. A total of 28 picks were made and cultured. Minipreps were done to prepare DNA. The DNA was then digested with EcoRI, EcoRV, and BamHI/XbaI, as before, and electrophoresed on a 1% agarose gel.

A culture of the positive clone was grown up overnight and miniprepped. A glycerol stock for 631A1 in the fetal brain library was made. We then sequenced the BamHI/XbaI cut fetal brain plasmid and one of the CEM PCR products. Since only a small segment of DNA can be sequenced at once, we digested with SstI and NotI to prepare the DNA binding site, and did an ExoIII timed deletion reaction to obtain varying fragment lengths. After the ExoIII reaction, vectors were re-ligated and transformed into bacteria by electroporation. Colony PCR was done to check them, and cultures were miniprepped. DNA was digested with PvuII and run on a 1% agarose gel. The plasmid was then sent for automated sequencing.

510C1

The same cDNA libraries—CEM and fetal brain—were initially screened for 510C1 by hybridizing P^{32} labeled cDNA probe with the original library membranes. However, after secondary screening, this proved ineffective, so new libraries were plated. Only the CEM library was plated. Plaque lifts were performed, and then the membranes were hybridized with the 510C1 radiolabeled probe. 15 picks for the secondary screening were made and plated.

SSAT Related Experiments

Induction of HL-60 with spermine: $4x10^4$ HL-60 cells were added to each of 8 wells with 100 μL of serum free media on a chamber slide. Spermine and SSAT antisense were added to the wells as shown in Table 1. The cells were incubated at 37 °C four hours, when fetal bovine serum was added to 10%. The cells were placed at 37 °C for an additional hour, then PMA was added to 3nM as indicated in Table 1. The cells were again placed at 37 °C for about 19 hours more.

Cells were resuspended and transferred to microwells. They were fixed in 4% formaldehyde for 10 minutes at room temperature, and then stained with anti-MacI antibodies (1:20) for one hour at 4 °C. Anti-mouse FITC conjugated secondary antibodies (1:100) were incubated with cells 30 minutes at room temperature. Cells were then cytospun onto glass slides for 5 minutes at 770 rpm.

Activity of SSAT antisense with PMA and no spermine in HL-60: $4x10^4$ HL-60 cells were added to 100 μL serum free media in 3 wells of a chamber slide. Though SSAT antisense should have been added to 200 μM in one well, there was only enough antisense to make it about 100 μM. The cells were incubated 4 hours at 37 °C when serum was added to 10%. After another hour of incubation, PMA was added to 3 nM in the antisense well and one other. The well with nothing but cells acted as the control.

After 8 hours incubation with PMA, cells were cytospun onto slides and fixed with 100% methanol 5 minutes. They were blocked with PBS/BSA 10 minutes and then stained with 1:100 anti-SSAT antibodies 30 minutes at room temperature. Goat anti-rabbit FIT C conjugated antibodies (1:100) were then added and incubated for 30 minutes at room temperature.

Determination of spermidine and putrescine toxicity in HL-60: HL-60 cells in culture were resuspended and counted with a hemacytometer. About $24x10^4$ cells were transferred to 1.5 mL serum free media. One plate was left as control, adding nothing. To four plates, 100 μM, 200 μM, 500 μM and 1 mM putrescine was added. To four other plates, 100 μM, 200 μM, 500 μM and 1 mM spermidine was added. These were incubated at 37 °C 4 hours, then serum was added to 10%. Cells were then taken at 19 hours and 48 hours of incubation for DAPI staining.

After 19 hours cells were spun down in a microcentrifuge, and fixed with 100% methanol for 5 minutes. They were stained with 1:30 DAPI and 1:500 hydroethidine in PBS for 7 minutes, and cytospun onto slides. After 48 hours, cells were cytospun onto slides first, and then fixed with 100% methanol for 5 minutes. Then they were stained with DAPI and hydroethidine as above.

Table 1. Variables for Induction of HL-60 with Spermine

Plate	PMA	Spermine	SSAT antisense
1	-	-	-
2	3 nM	-	-
3	-	2 μM	-
4	-	4 μM	-
5	3 nM	2 μM	-
6	3 nM	4 μM	-
7	3 nM	2 μM	200 μM
8	3 nM	4 μM	200 μM

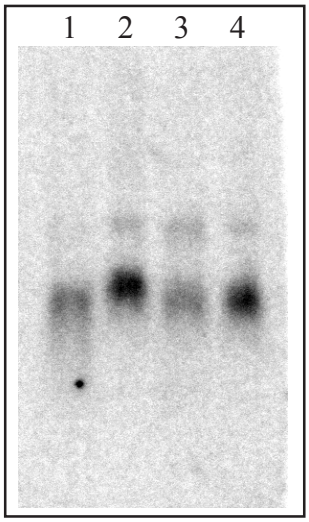

Figure 3. Northern blot hybridized with 631A1 probe to test expression in different DNA libraries. Lane 1 is 631A1expression in the prostate PG-3 library. Lane 2 is expression in the CEM library. Lane 3 is expression in the Breast AC 565 library. And lane 4 is 631A1 expression in HL-60. Since expression was comparatively higher in the CEM library, that is the library we chose to screen for 631A1.

The spermidine toxicity experiment was repeated with about $44x10^4$ cells per plate at concentrations of 3 μM, 10 μM, 30 μM and 100 μM. They were stained by spinning in a microcentrifuge first, fixing with 4% formaldehyde, adding DAPI and hydroethidine as before, and then cytospinning.

ODC probe synthesis: Ornithine decarboxylase cDNA probe was made from ODC mRNA using a reverse transcriptase reaction. In a PCR cycler, the mRNA plus 1x RT buffer, 20 μM dNTPs, 0.2 μM ODC reverse primer and RNAse-free treated water was held at 68 °C for 10 minutes. The block cooled to 37 °C where the cycle was paused and 0.5 μL MMLV reverse transcriptase was added to the reaction. It was then held at 37 °C for 1 hour, heated to 75 °C for 5 minutes, and then stored at 4 °C infinitely. The RT reaction was then amplified using Taq polymerase by normal PCR. The probe was used to look at ODC expression during PMA induction of HL-60.

RNA gel of 3-2 cells: RNA was prepared for 3-2 and HL-525 cells that were treated with PMA. A 1% agarose gel with MOPS and formaldehyde was run, and the gel was transferred onto a membrane by capillary action.

RESULTS

631A1

Three DNA libraries were tested for expression of 631A1 with a northern blot. The expression in HL-60 behaves as a positive control. The CEM library demonstrates the highest expression (Figure 3). When the CEM and fetal brain libraries were plated out, lifted, and membranes were hybridized with 631A1 probe, the fetal brain library yielded fewer, yet stronger hits. The CEM library had more hits that had weaker signal. Background was very low. In secondary screening, the signal, when present, was very strong.

The secondary CEM picks that were PCRed showed three promising bands when they were electrophoresed. The fetal brain plasmid picks digested with BamHI and XbaI yielded fragments around 1kb, which was the expected cDNA length for 631A1. Based on this, we isolated DNA from picks 6 and 9, two of the CEM PCR products, and picks 1 and 5, two of the digested fetal brain plasmids (Figure 4). The PCR products were digested with EcoRI to make sticky end ligation possible. Pick number 6 had an internal EcoRI restriction site, suggesting that perhaps there were two library inserts in that clone, since the smaller fragment was then very close in size to that expected (Figure 5).

The blue-white screening yielded only one clone positive for the expected 631A1 insert size of 1kb out of forty picks. We also tried ligating again into new vector, and plating on S-gal plates (brown-white screening media from Sigma), but this did not yield any positive results either. Even so, we continued by sequencing the positive BamHI/XbaI digested clone and the CEM PCR product number 9 that was the expected size.

Initial sequencing yielded no results for the plasmid, and only sequence from one primer in the CEM fragment. Trouble-shooting indicated that there was little to no DNA in the fetal brain plasmid prep. The T7 primer was found to be ineffective for the CEM fragment sequencing. A second miniprep and new T7 primer then yielded about 300bp of 631A1 sequence. The 3' end of the cDNA sequence matched the sequence of the 631A1 probe.

The ExoIII deletion reaction worked as expected, deleting about 200bp per 30 seconds. This can be seen on the step-like gel of the eight time points. The first time point has the largest fragment; the last time point has the smallest fragment (Figure 6).

PCR of the time points from the ExoIII reaction yielded strange results. The PCR only work for the 2 minute time point, and all four picks resulted in fragments of differing sizes. Digestion of miniprepped

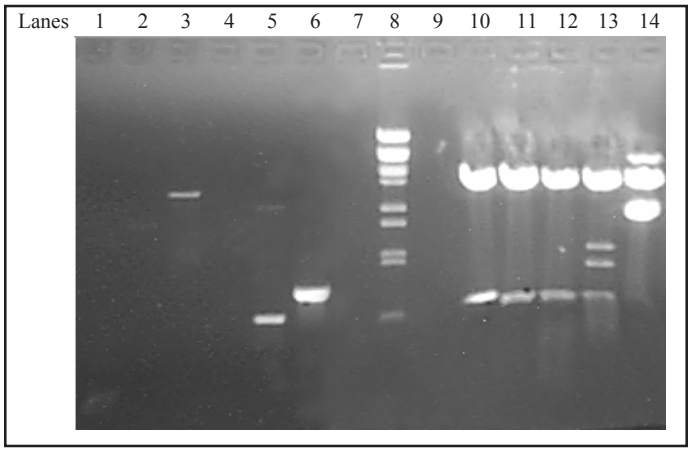

Figure 4. Agarose gel of CEM library PCR products and BamHI/XbaI digested fetal brain library plasmid. Lanes 1-6 contain the PCR products from the CEM library. Lane 3 and lane 6 contain picks 6 and 9, which were arbitrarily chosen to be subcloned. Lane 8 is the high molecular weight marker λ–BstE II. Lanes 10-14 are fetal brain plasmids digested with BamHI and XbaI. Lanes 10 and 14, or picks 1 and 5 were also chosen to be subcloned. All of these clones were isolated from 631A1 secondary screening.

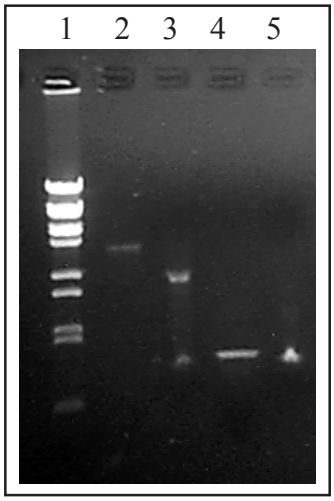

Figure 5. Results of EcoRI digestion of CEM picks 6 and 9. Lane 1 is the high molecular weight marker λ–BstE II. Lane 2 is pick #6 PCR product. Lane 3 is #6 digested with EcoRI. Notice that it is split into two bands, and that the smaller is nearly identical to #9 in size, which is around 1kb. Lane 4 is pick #9 PCR product, and lane 5 is #9 digested with EcoRI.

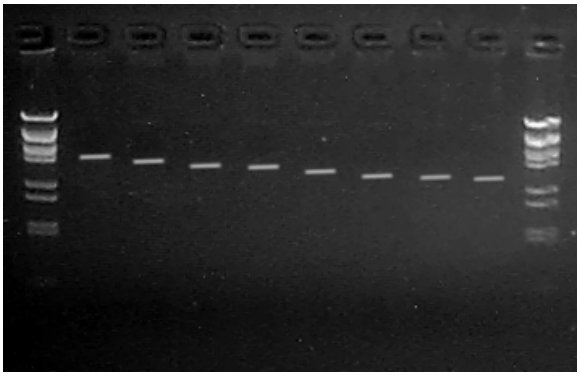

Figure 6. Agarose gel of Bluescript plus the 631A1 insert at 8 time points of the ExoIII deletion reaction. The length of insert becomes progressively smaller as the enzyme deletes about 400bp per minute. The loose end of vector DNA was protected by a 3' overhang. The samples were run with the λ–BstE II marker.

DNA with PvuII yielded bands of the same size for all time points and all were too large. Only the control looked correct in size.

510C1

Hybridization of the 510C1 probe to the original library membranes resulted in much higher background than the 631A1 screening. The strongest hits failed to produce colonies or plaques on the secondary plates. Signal after the secondary membranes were hybridized were also very weak. The picks that were made from the secondary plates were all determined to be negative for 510C1 insert when there was no DNA amplified by PCR. We then plated and hybridized new CEM libraries. The newly plated library yielded very strong signal with extremely low background.

SSAT Experiments

Induction of HL-60 with spermine: When spermine was added to the media in concentrations similar to that naturally in the cell, PMA induction of differentiation in HL-60 was blocked. Table 2 indicates the level of cell attachment and MacI expression. PMA induced cells had both positive attachment and MacI expression. Concentrations of spermine resulted in no attachment of MacI expression. PMA plus spermine resulted in some attachment with 2 μM spermine. Minimal attachment was seen at 2 μM spermine with PMA and SSAT antisense.

Activity of SSAT antisense with PMA: This experiment tests whether the SSAT antisense functions as it is expected to. Here we saw induction of SSAT, and not very much blocking of SSAT expression. The experiment was repeated with new antisense and only 4 hours of PMA induction, and SSAT expression was blocked.

Spermidine and putrescine toxicity: Cells appeared healthy at all concentrations of putrescine, therefore putrescine is not toxic to HL-60 cells. Spermidine caused a significant number of cells to be apoptotic at 100 μM, so the experiment was repeated. At 3 μM, 10 μM and 30 μM, cells appeared healthy, although it was apparent that there were more apoptotic cells at 30 μM than the lower concentrations. Cells once again were apoptotic at 100 μM spermidine.

ODC probe: Hybridization of the ODC probe to a RNA membrane for PMA induced HL-60 cells showed normal levels of ODC expression at 3 hours, but decreasing levels of expression after 8 hours of induction.

3-2 cell blot: Hybridization of the blot for 3-2 and 3-30 cells with SSAT probe showed expression of SSAT at 8 hours of PMA induction. There was no induction of SSAT in the HL-525 control.

DISCUSSION & CONCLUSIONS

The rationale behind spending time screening for desired genes and sequencing is as follows: Although the human genome is supposedly sequenced, there is still a significant percentage of unknown genes, and there is little knowledge about the function of many genes. By screening a library with a cDNA probe, we are able to isolate the gene. Then sequencing enables us to learn a great deal about the gene. Similarities to other known sequences may infer the gene's function. The presence of known domains in the sequence might hint at how the gene is regulated. Finding the open reading frames would make it possible to determine the amino acid sequence of the gene. Finally, having the gene sequence enables one to determine the 3' antisense. Obtaining the antisense is important for functional studies of the gene because it blocks expression of the gene, essentially creating a knockout model cell.

In our experiments described here, we initially attempted subcloning the 631A1 insert into Bluescript Sk(+) instead of immediately sequencing the positive clone so that we could do the ExoIII deletion reaction. This would allow us to do several sequencing reactions in parallel and obtain the entire gene sequence manually instead of having to make a new primer for the next 300 bp region in the gene each round. While the ExoIII reaction worked, some kind of plasmid contamination made transformants impossible to find easily. This encouraged us to discontinue manual sequencing efforts and send the positive clone to the University of Chicago for automated sequencing. We had already confirmed that we had the gene we were looking for because the 3' end that was manually sequenced matched that of the probe. The 5' sequenced end had identity with some sequences in the database, which is strong evidence that the cDNA sequence is full.

Learning from our experience with 631A1, after a positive clone is found and isolated for 510C1, we will most likely forego subcloning and manual sequencing, and send it for automated sequencing directly, using subcloning and manual sequencing only for confirmation.

Thus far, experiments with SSAT have shown that the antisense is capable of blocking SSAT expression, and so is capable of blocking differentiation in HL-60 by itself. Spermine alone, however is unable to block differentiation. The combination of antisense and spermine together is also capable of blocking macrophage differentiation. This, along with the knowledge of toxicity levels of polyamines in the cell will facilitate planning future functional studies of SSAT and the polyamines.

Expression studies have also yielded some interesting findings. SSAT expression in 3-2 and 3-30 cells, but not in HL-525 indicate that PKC-β may play a role in SSAT regulation. 3-2 and

Table 2. Spermine Induction Results

	c	p	2uM s	4uM s	2uM sp	4uM sp	2uM spa	4uM spa
cell attachment	-/+	+	-	-	+/-	-	-/+	-
MacI expression	-	+	-	-	-	-	-	-

c = control p = 3nM PMA s = spermine a = SSAT antisense

3-30 are cell lines derived from HL-525, and are stably transfected with a plasmid coding for PKC-β. There is also a transcription factor called Nrf2 that is expressed after 3 hours of PMA induction in HL-60 (Gavin, 2001). This might also play a role in regulating SSAT, because SSAT is not expressed until 8 hours.

The isolation and sequencing of 631A1 and 510C1 will be completed soon. Obtaining the antisense sequence will enable us to learn much more about the function of these currently unknown genes. It will be interesting to discover where in the scheme of macrophage differentiation these new genes fit in. We can only hope that it will make what we now know about differentiation even more clear. In addition, knowing how SSAT is regulated is the beginning of a new understanding of polyamine regulation in the cell. This could lead to identification of new targets for cell growth and differentiation control, giving us yet another tool for finding treatments for cancer.

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