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Heme Initiates Changes in the Expression of a Wide Array of Genes during the Early Erythroid Differentiation Stage

Yonghua Zhu, Thomas Hon, and Li Zhang[1]
Department of Biochemistry, NYU Medical Center, 550 First Avenue, New York, New York 10016

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Heme is central to oxygen sensing and utilization in all living organisms. It directly regulates numerous molecular and cellular processes for systems that sense or use oxygen. In mammals, heme plays an indispensable role in erythroid cell differentiation. To investigate heme regulatory functions, we identified, by differential display, and confirmed, by quantitative RT-PCR and Northern blotting analysis, the genes whose expression is altered by heme during the early stage of K562 cell differentiation. These include genes encoding a GAP-associated p62 protein, histone H2A.Z, a subunit of the small nuclear ribonucleoprotein complex, and the chaperonin Tcp20, and a cellular immediate-early-response gene. The results suggest that heme initiates changes in key factors that control a wide array of processes ranging from cell cycle and Ras signaling to chromatin structure, splicing and protein folding. These key factors might act together to mediate heme action, which is critical for erythroid cell differentiation.

Heme is a central factor in oxygen sensing and utilization in all living organisms ranging from bacteria to humans. It permits oxygen binding and serves as a redox-reactive center in numerous enzymes and protein complexes involved in using oxygen and controlling oxidative damages. Remarkably, heme also plays important regulatory roles in fundamental molecular and cellular processes that sense or use oxygen (1,2). For example, in yeast, heme, through the heme activator protein 1 (HAP1) (3,4), directly mediates the effects of oxygen on the expression of genes encoding functions required for respiration and for controlling oxidative damages (5). In mammals, heme controls a wide array of molecular and cellular processes including cell differentiation, transcription, translation, protein translocation and assembly (1). For example, it stimulates the growth and differentiation of erythroid, adipose, and nerve cells (1,2,6). In particular, heme is a potent inducer of erythroid differentiation of the human erythroleukemia K562 cells; it strongly induces the synthesis of hemoglobin in these cells (7-9). Therefore, the human K562 cells are used in studying heme regulatory functions. However, the regulatory genes or proteins that mediate heme action in mammalian cells remain unidentified.

In this report, to gain insights into how heme exerts its regulatory effects, we identified genes that are differentially expressed in heme-treated and untreated K562 cells. Previous experiments showed that genes encoding globin chains and enzymes involved in heme synthesis are strongly induced in the late induction stage after 48 hours of heme treatment (7,8,10). We envisioned that many key regulatory factors would be turned on during the early induction stage while genes encoding globin chains and other erythroid-specific functions would be turned on in the late period, as in neuronal differentiation and viral lytic cycles (11,12). Therefore, to identify specifically those regulatory genes that are potentially important for heme induction of erythroid differentiation, we isolated clones whose expression was changed at 4 and 8 hours of heme treatment by differential display. We found that a wide spectrum of genes, including those encoding a GAP-associated protein p62, chaperonin Tcp20, histone H2A.Z, and a subunit of the ATPase proton channel, were differentially expressed in the early stage of heme induction. These results suggest that heme initiation of erythroid cell differentiation may involve the cooperative actions of numerous regulatory factors with diverse functions.

MATERIALS AND METHODS

Cell culture and heme treatment. The human chronic myelogenous leukemia K562 cells (13) were maintained in RPMI 1640 media with 10% fetal bovine serum (FBS). After pre-culturing for 24 h, 50

[1] To whom correspondence should be addressed. Fax: (212) 263-8166. E-mail: zhangl02@mcrcr0.med.nyu.edu.

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μM of heme (7,8,10) was added, and the cells were incubated further for an additional 4 and 8 hours. These early time points were chosen to identify regulatory genes critical for the initiation of erythroid differentiation rather than non-regulatory genes such as genes encoding globin chains and enzymes for heme biosynthesis, which are induced at later time points (10,14).

RNA isolation. Total RNA from heme treated and untreated K562 cells was extracted by using the single step guanidium thiocyanate phenol-chloroform extraction procedure as described (15). For differential display analysis and quantitative RT-PCR, possible DNA contamination was removed by treating RNA with RNase-free DNase I for 30 min at 37°C.

Differential display. All oligonucleotides used were purchased from GenHunter Corp (16,17). mRNA differential display was performed according to the method described by Liang and colleagues (16-18), with slight modification. Briefly, 0.2 μg of DNA-free total RNA was reverse-transcribed by using H-T11N primers (16-18). 1/10 of reverse-transcribed mixture was directly used for PCR amplification in the presence of α-^{33}P dATP in a 20 μl reaction volume. The cycling parameters are as follows: 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds for 40 cycles, followed by 72°C for 5 minutes. The amplified cDNAs were separated on a 6% DNA sequencing gel. AmpliTaq DNA polymerase was purchased from Perkin-Elmer Cetus Corp. Each set of reactions was independently repeated at least three times, and only those reproducible bands that are present in either heme-treated or untreated cells were recovered from dried gels and reamplified in a 40-cycle PCR using corresponding primers.

Cloning and sequencing of cDNAs. Reamplified cDNA fragments were cloned into the pGEM-T vector using pGEM-T vector system (Promega). Sequencing of the cloned cDNA fragments with either T7 or SP6 primer was performed by the ABI PRISMTM Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer) and the Automated DNA sequencer (Perkin Elmer ABI PRISMTM model). Comparison of DNA homology with GenBank and the EMBL database was performed by using BLAST.

RT-PCR quantitation. RT-PCR analysis was carried out as described previously (19). Primers used for PCR amplification are as follows: GAPDH-F 72-92 GAA GGT CGG AGT CAA CGG ATT, GAPDH-R 606-586 AGT GAT GGC ATG GAC TGT GGT; HRI-F 852-872 CTC AGG TGG ATT TGG CCA AGT, HRI-R 1538-1518 ATG ATG CCA TCC CGT AGG TCT; 1-F 33-53 TTA TAT ATG TCT GAC CCC AGG, 1-R 351-331 GAA CCA TGC CTG TTT ACA GTA; 4-F 99-109 GGC ATA AAC ACT GAC GTC CCT, 4-R 479-459 GGT CTT GGG AAT GTG AAC ACA; 5-F 294-314 GAA CAG AGA ACC TGT ACA GCT, 5-R 685-665 AAT GCT TAG CTC TCA CTG TGG; 6-F 246-266 TGT GCT ACT CAA CTC AGG AGA, 6-R 678-658 AGT AGG CCA GCG GTAATC TTC; TCP20-F 1051-1071 AGA GGC TGA CTC TTG CTT GTG, TCP20-R 1660- 1640 CCA GCT CGC ATG ATC TCA TCA; P62-F 638-658 GGA CCA CAA GGG AAT ACA ATC, P62-R 1066-1046 CCT TAC TGG TGT ACC ACG TAC; H2A.Z-F 51-71 TGG CAC CGA TTA GCC TTT TCT, H2A.Z-R, 465-445 CCA ATC AGA GAT TTG TGG ATG; RIBB-F 304-324 GCT TAT AAG ATC AGG ATC CGT, RIBB-R 689-669 TTC CTA CGT ATC TCC CGT ACA; 11-F 378-398 GAG CAG ATC ATG AAG TCC ATC, 11-R 783-763 CTG CGT ATG TGT CAG GCT GTT; 12-F 32-52 TTA TTT CCT GAG CAT GCT GAC, 12-R 411-391 CTT CGA AAT TGT ACT CCC TGC. The primers were designed to have a GC content close to 50%. These primer pairs contain coding sequences and span at least two exons when possible. Briefly, 2 μg of each DNA-free total RNA sample was reverse transcribed into first-strand cDNA in a 20 μl reaction using the Reverse Transcription System and Oligo-$dT_{12\text{-}18}$ (GIBCOBRL) according to the manufacturer's instructions. 1/20 of cDNA from the 20 μl reactions was used for each PCR reaction. Each PCR reaction contained 1× PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase, two pairs of primers (one pair for target gene, the other for GAPDH gene used as an internal control) and 2 μCi ^{32}P-end labeled 5′-primer (1500 Ci/mmol). The optimal conditions for amplification (the proportion between the two pairs of primers, temperatures, and cycle numbers) were experimentally determined and subsequently used as follows: 95°C for 1 minute, 56°C for 45 seconds, 72°C for 1 minute for 20 cycles, followed by 72°C for 10 minutes. The PCR products were loaded on a 5% polyacrylamide gel in 1× TBE. After electrophoresis, the gels were dried and exposed on a Storage Phosphor Screen (Molecular Dynamics). The DNA bands were visualized and quantified by using the PhosphoImage system (Molecular Dynamics). The mRNA amount of a target gene was normalized by the equation: intensity of the band representing the target mRNA/ intensity of the band representing GAPDH mRNA.

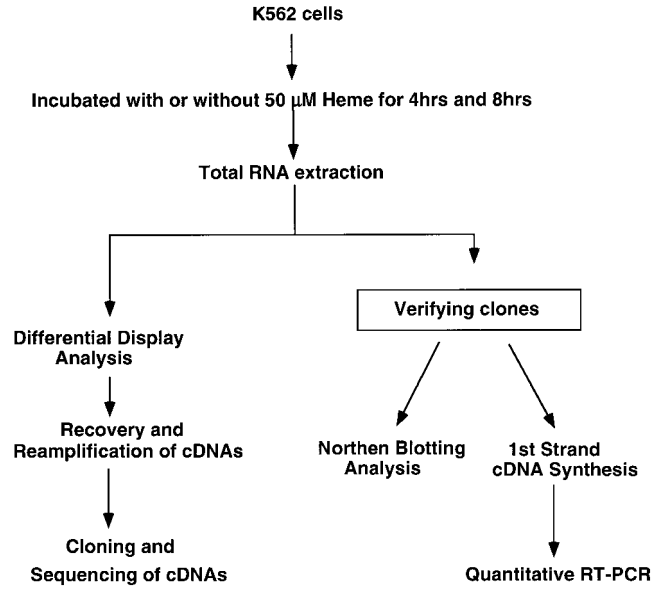

FIG. 1. A scheme illustrating the strategy used to identify and verify the differently expressed clones.

Northern blot analysis. 15 μg of total RNA was separated on 1% agarose gels containing 2.4% formaldehyde and transferred to Hybond-XL nylon membrane (Amersham). RNA was immobilized onto membranes in a vacuum oven at 80°C. cDNAs obtained from RT-PCR amplification or clones from differential display were used as probes for Northern blot analysis. Probes were labeled with α-^{32}P dCTP by PCR. After overnight hybridization, membranes were washed in 2× SSC (1× SSC = 44.6 mmol/L sodium chloride, 5 mmol/L trisodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) solution at room temperature for 15 minutes twice, 0.5× SSC, 0.1% SDS at 68°C for 15 minutes twice, and exposed on a Storage Phosphor Screen (Molecular Dynamics) for overnight. The RNA bands were visualized and quantified by using PhosphoImage system (Molecular Dynamics).

RESULTS

Differential Display of mRNAs from Heme-Treated versus Untreated K562 Cells

To identify the differentially expressed genes in the early heme induction stage of K562 cells, we carried out mRNA differential display of K562 cells treated with heme for 4 or 8 hours versus untreated cells. The strategy for identifying and verifying these differentially expressed genes is outlined in Figure 1. Specifically, we treated K562 cells with 50 μM heme as de-

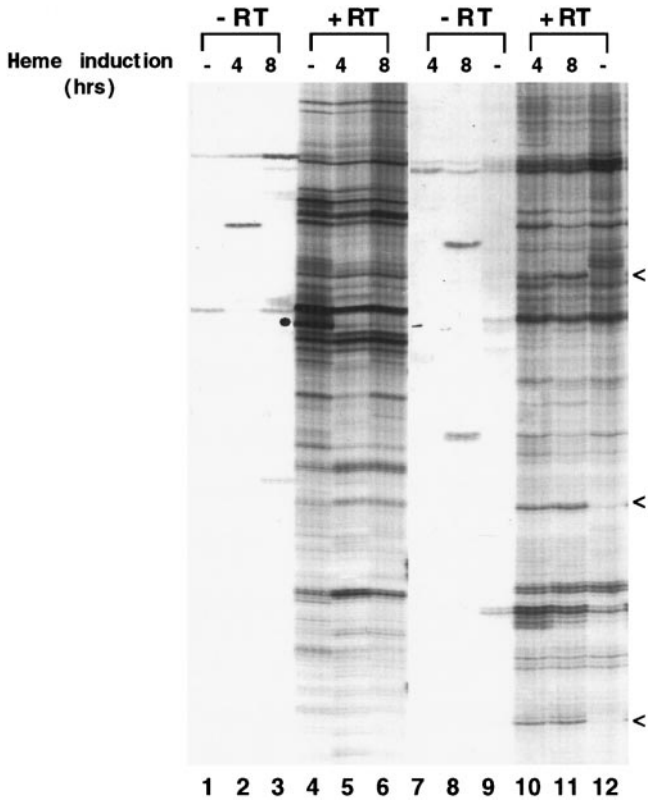

FIG. 2. Examples of results from differential display analysis. mRNA samples from K562 cells treated with heme for 4 (lanes 2, 5, 7 and 10) or 8 hours (lanes 3, 6, 8 and 11) and untreated cells (lanes 1, 4, 9 and 12) were prepared and subjected to reverse transcription (lanes 4-6 and 10-12) using the anchored H-T11C (lanes 4-6) or H-T11A (lanes 10-12) primer (GenHunter Corp). As controls, the same mRNA samples were used for PCR amplification without reverse transcription (lanes 1-3 and 7-9). Then, the cDNAs were PCR amplified using the corresponding anchored primer used in the reverse transcription and the arbitrary H-AP4 (lanes 1-6) or H-AP5 (lanes 7-12) primer (GenHunter Corp) (16,17). After amplification, the products were analyzed on sequencing gels.

scribed previously (8,14,20) for 4 or 8 hours. Under this heme concentration, more than 50% of the cells expressed a high level of hemoglobin after 2-3 days of heme treatment, as shown previously (8,14,20). mRNA samples from heme-treated and untreated cells were prepared and reverse transcribed by using one of the three possible anchored oligo-dT primers (H-T11G, H-T11C, and H-T11A from GenHunter Corp) (16,17). Then, the cDNAs were PCR-amplified by using the corresponding anchored primer used in the reverse transcription and another arbitrary primer (GenHunter Corp) (16,17). After amplification, the products were analyzed on sequencing gels. As shown in Figure 2, most bands were present in samples from both heme-treated and untreated cells (compare lanes 4-6 and lanes 10-12). However, certain bands were present only in the samples of heme-treated cells (see bands marked by arrow heads in lanes 10 and 11), while other bands were present only in untreated cells (for an example, see the band marked by a dot in lane 4). Those bands present only in samples of heme-treated cells may represent genes induced by heme while those present only in samples of untreated cells may represent genes that are suppressed by heme. Those bands present in only heme-treated or untreated cells were cut out from the gel, reamplified, and cloned into a sequencing vector. To rule out false positives, each reaction with one set of primers was repeated three times, and only those repeatedly found to be differentially expressed were isolated and further analyzed.

We used three anchored primers, paired with 32 arbitrary primers, and we isolated 59 bands from the sequencing gels. Twenty cDNAs were eventually cloned and sequenced, and 15 of these had identical clones. In sum, we initially identified 12 distinct genes that were differentially expressed in heme-treated K562 cells versus untreated cells (Table 1; clone 3 was shown to be unaffected by heme by Northern blotting analysis, see Figure 5). Three of the genes (clones 6, 11 and 12) were repressed by heme while seven were induced by heme (clones 1-2, 4-5 and 7-9). One of the clones (clone 10) was found to be repressed at 4 hours but induced at 8 hours. Ten of these 12 genes were found to be homologous/identical to previously identified genes (Table 1), and they encode a wide spectrum of proteins ranging from chaperonin to GAP-associated proteins.

Confirmation of the Differentially Expressed Genes by Quantitative RT-PCR

To confirm that the expression of the identified genes is indeed altered by heme, we carried out RT-PCR and Northern blotting analyses, as outlined in Figure 1. Because the cloned fragments from differential display are the 3′ end untranslated regions and are often unstable, they are not usually used as probes in quantifying mRNA. To overcome this problem, mRNAs identical to known genes were analyzed by quantitative RT-PCR analyses (19). To ensure that the analyzed mRNA was indeed from the specific genes, we designed primer pairs that contain coding sequences, spanning two exons when possible. These primers all have GC contents similar to the primers for the control GAPDH cRNA. All our primer pairs yielded PCR products with the exact length as predicted from the known sequences. To determine the optimum conditions for RT-PCR analysis of each gene, we carried out the following tests. First, to ensure that both the analyzed cDNA and the GAPDH cDNA are amplified at about the same efficiency, we determined the ratio of the pair of primers for the analyzed cDNA to the pair of primers for the GAPDH cDNA. Because the amount of each specific mRNA or cDNA was different, the primer ratio for each analyzed gene was determined separately. Then, we

TABLE 1

Summary of Clones Identified by Differential Display in Heme-Induced K562 Cells

Clone	Effect of heme	Homology[a]	Accession no.
1	↑	Homo sapiens cDNA clone (163/164:99%)	H93366
2	↑	No homology found[b]	AF102151
3	none	No homology found[c]	AF102150
4	↑	Soares multiple sclerosis 2NbHMSP Homo sapiens cDNA clone (312/315:99%)	N64787
5	↑	Human small nuclear ribonucleoprotein Sm-D autoantigen (181/182:99.4%)	J03798
6	↓	Human ATL-derived PMA responsive gene (116/118:98%)	D90070
7	↑	Human chaperonin protein Tcp20 (65/66:98%)	L27706
8	↑	GAP-associated tyrosine phosphoprotein p62 (72/72:100%)	M88108
9	↑	Human histone H2A.Z gene (211/214:98.5%)	X52317
10	↓↑	Human small G protein RIBB of the Ras family (137/143:96%)	U78165
11	↓	Vacuolar H^+-ATPase proton channel subunit (233/238:98%)	M62762
12	↓	Human ubiquinol-cytochrome C reductase complex 7.2KD protein (275/276:99%)	AA534055

[a] All mismatches are due to uncertain nucleotide sequences.

[b] TTTTTTTTTTTCAAGTGAATTACCTTACCAAGAAGTGTCAATCAATTTAGCAGCAAAATAAGCCAACGTAGCCAGAGGGAGCAGAGGGTCTGGAACTGTGGCTCCTGAACCTGTCTGGTCATTAAATCACCTGGT.

[c] ACGCAACCACAGAACAATTATCAAAAGCTTGGAAACTAACATTGATACAATACTTTAATCTACATACCTCATTCAGATTTCACCAGTTGTCCTAATAATGTAATAAAATTATTTCTGGAAAAAAAAAAA.

carried out a kinetic study to determine the number of cycles appropriate for quantitative PCR analysis. To quantify the amount of mRNA, the number of cycles should be enough to permit the detection of signals from both the analyzed mRNA and the control GAPDH mRNA, but before the amplification of one or both cDNAs reaches saturation. Figure 3 shows how the cycle numbers were chosen. Using the same amount of RNA and amplification conditions, clone 5 and GAPDH mRNAs/cDNAs were coamplified for 16 to 24 cycles. Clearly, within these cycle numbers, the amount of both products increased proportionately with the cycle number, and the ratio between the two genes remained the same, suggesting that amplification of the cDNAs did not reach saturation. Considering also the intensity of the bands, we used 20 to 22 cycles for all analyzed genes shown in Figure 4.

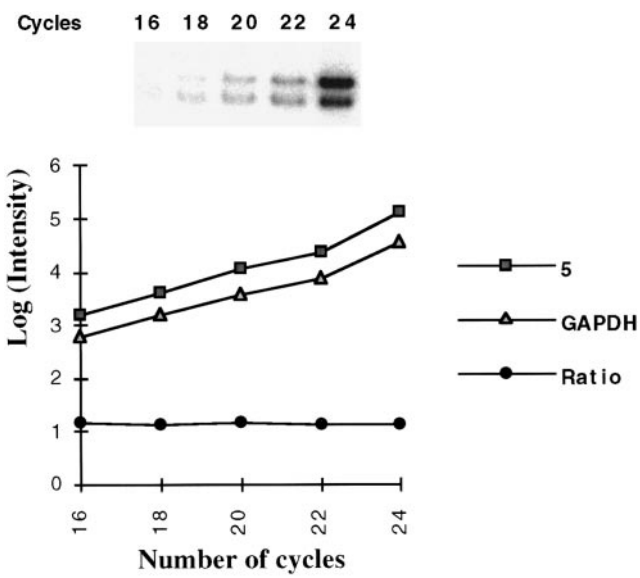

FIG. 3. Determination of optimum conditions for quantitative RT-PCR. Reverse transcribed cDNA was coamplified with primers for the clone 5 and GAPDH genes for 16 to 24 cycles. The intensity of each band was quantified by ImageQuant on a PhosphorImager. Ratio = log(Clone 5)/log(GAPDH).

First, we tested the quantification profile of the heme-regulated eIF2-α kinase (HRI kinase), which is known to be induced by heme (21,22). As shown in Figure 4, we coamplified mRNA/cDNA of HRI kinase and GAPDH. For each mRNA sample, the ratio of HRI kinase to GAPDH was calculated and shown. The result shows that HRI kinase was induced 2- to 3-fold by heme, as reported previously (22). Then, we quantified the relative mRNA amounts of all identified clones identical to known genes. In summary, we found that mRNAs of clone 1, clone 4, Tcp20, p62, and H2A.Z were induced 2- to 3-fold by heme after both 4 and 8 hours of heme treatment (Figure 4). Clone 5 was induced by heme after 8 hours of heme treatment (Figure 4). mRNAs of clones 6, 11, and 12 were down-regulated 2- to 3-fold by heme (Figure 4). Interestingly, mRNA of clone 10 (RIBB) was first down-regulated 2-fold at 4 hours and then up-regulated 2-fold at 8 hours (Figure 4).

Confirmation of the Differentially Expressed Genes by Northern Blotting Analysis

For those genes not identical to known genes (clones 2 and 3), we used Northern blotting analysis to determine the effect of heme on their expression. We used the GAPDH mRNA as a control and detected mRNA of Tcp20 for comparison with RT-PCR analysis. As shown

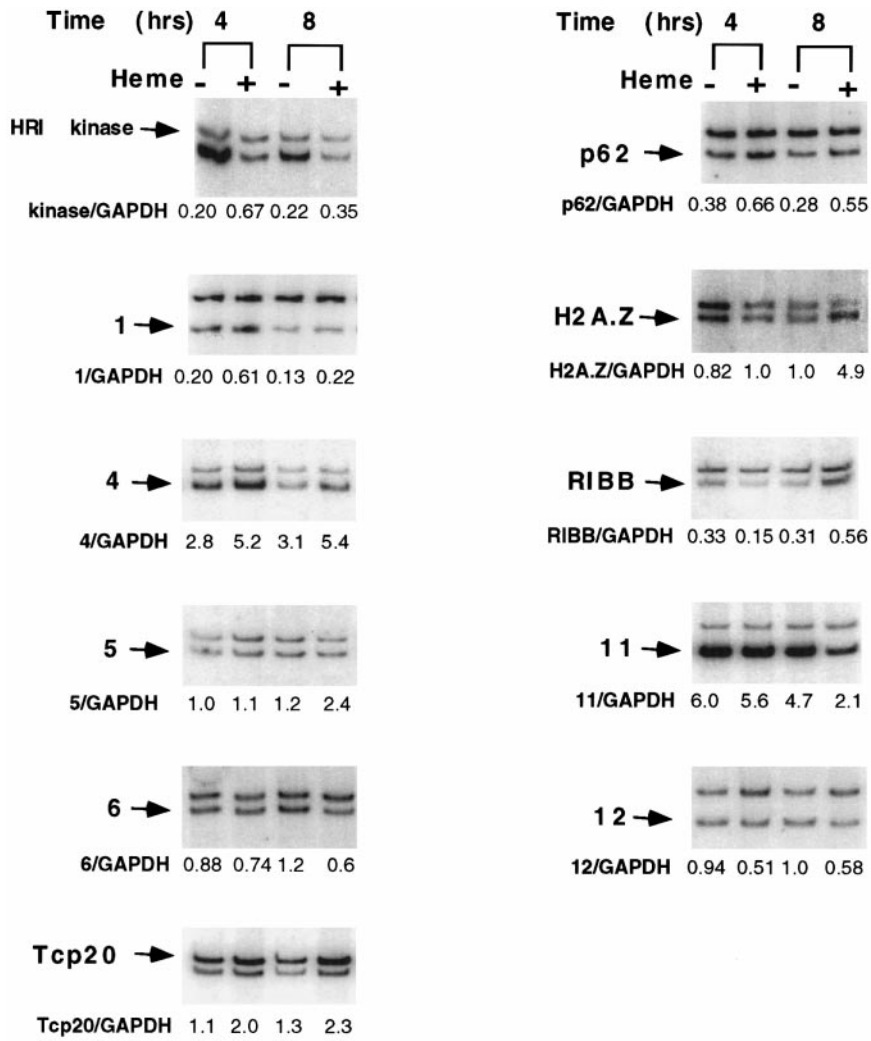

FIG. 4. Quantitative RT-PCR analysis of differentially expressed clones identical to known genes. Reverse transcribed cDNAs from heme-treated and untreated K562 cells at indicated time points was coamplified with target gene and GAPDH primers, labeled with ^{32}P. The PCR product was analyzed on 5% polyacrylamide gel, and the intensity of each band was measured by ImageQuant on a PhosphorImager. Ratio = intensity of target gene/intensity of GAPDH (the ratio is shown under each lane). Shown are the data for the heme-regulated eIF2α kinase, a positive control, clones 1, 4-6, Tcp20, p62, H2A.Z, RIBB, clones 11 and 12. The positions of the analyzed genes are marked by arrows; the unmarked band in the gels represents GAPDH. These experiments were repeated independently for three times, and the variation was generally within 30%.

in Figure 5, Tcp20 was induced about 2-fold by heme at both 4 and 8 hours of induction, consistent with the result obtained from RT-PCR analysis. Northern blotting analyses of other clones, such as H2A.Z and p62, also gave the same results as RT-PCR (not shown). Clone 2 was largely unaffected at 4 hours but induced 2- to 3-fold at 8 hours, while clone 3 was not affected at both 4 and 8 hours of heme treatment.

DISCUSSION

In this report, to identify those regulatory genes that may play roles in heme induction of erythroid differentiation, we carried out mRNA differential display of K562 cells treated with heme for 4 or 8 hours versus untreated cells. We identified 11 distinct heme-regulated genes: Seven are induced; three are repressed; one is repressed at 4 hours of induction and induced at 8 hours of induction (Table 1). These genes encode a host of proteins with seemingly divergent functions. Most of these genes encode regulatory functions, supporting our original idea that regulatory genes would be turned on during the early stage of heme-induced differentiation.

The first class of heme regulated genes identified are those involved in Ras signaling, including the GAP-associated tyrosine phosphoprotein p62 (clone 8, Table 1) (23,24) and the small G protein of the Ras family,

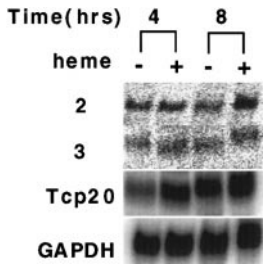

FIG. 5. Northern blot analysis of differentially expressed clones with no homology to known genes. Total RNAs from heme-treated and untreated K562 cells at 4 and 8 hours were probed with ^{32}P-labeled amplified cDNA. Shown are data for clones 2 and 3, and GAPDH for control, and Tcp20 for comparison.

RIBB (clone 10, Table 1) (25). p62 is induced by heme at both 4 and 8 hours of heme treatment (Figure 4), while RIBB is repressed by heme at 4 hours but induced at 8 hours. Interestingly, p62 is also induced in differentiating keratinocytes immediately following calcium addition (23). Ras and GAP-associated proteins are often positioned downstream of cell surface receptors and upstream of a cascade of cytoplasmic kinases that in turn regulate the activity of a host of transcription factors (25). Perhaps p62 and RIBB can act together to control the activity of transcription factors that activate transcription of genes encoding globin chains and other erythroid-specific functions in response to heme. The third heme-induced gene is H2A.Z, a H2A isoprotein, which represents about 5% of the total H2A in vertebrates (26-28). H2A.Z is synthesized at a constant level throughout the cell cycle (26). It was suggested that the function of H2A.Z is to substitute for S phase linked histones in chromatin for controlling transcription (29-32). In heme-induced erythroid K562 cells, it may be involved specifically in remodeling the chromatin structure of the erythroid-specific genes and promoting their transcription. The fourth heme-induced gene is the human small nuclear ribonucleoprotein Sm-D autoantigen, which is a subunit of the small nuclear ribonucleoprotein complexes (snRNPs) (33). We imagine that this protein might have a role in erythroid-specific splicing. The fifth heme-induced gene is the molecular chaperonin Tcp20, a subunit of the eukaryotic cytosolic chaperonin complex, which is highly conserved from yeast to humans (34,35). Previously, it was shown that heme induces the expression of stress genes, such as HSP70 and HSP90, in K562 cells (36). Tcp20 might be another stress gene induced by heme. It is not clear what functions the other heme-induced clones 1, 2 and 4 may have in erythroid cell differentiation because no homology was found for clone 2, and no functional information is available for clones 1 and 4.

Three genes were repressed by heme. The first one is the adult T-cell leukemia (ATL) PMA responsive gene (37). It is a member of the cellular immediate-early-response genes whose expression is rapidly induced by phorbol-12-myristate-13-acetate in a wide array of cells including T cells and embryonic cells (37). Previous data suggest that it plays a role in cell cycle reentry (G_0/G_1 transition) during cell proliferation. Thus, this gene should be repressed for differentiation to occur when K562 cells are induced by heme. The other two heme-repressed genes are clones 11 and 12, encoding a vacuolar H^+-ATPase proton channel subunit (38) and a subunit of the mitochondrial cytochrome c reductase, respectively. These genes are repressed by heme, probably because differentiated red blood cells do not need vacuolar and mitochondrial functions.

Our studies here show that a host of genes are affected in the early stage of heme-induced erythroid cell differentiation. Most of these genes encode regulatory functions, ranging from those involved in cell proliferation and Ras signaling to those involved in chromatin structure and splicing. These results indicate that heme may affect a wide spectrum of regulatory factors, not just transcription factors, and that heme-induced erythroid cell differentiation involves changes not only in transcription, but also in cellular signaling pathways and in splicing.

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