Identification of mRNAs Differentially Expressed in Quiescence or in Late G1 Phase of the Cell Cycle in Human Breast Cancer Cells by Using the Differential Display Method

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ABSTRACT

Background: The decision for a cell to enter the DNA synthesis (S) phase of the cell cycle or to arrest in quiescence is likely to be determined by genes expressed in the late G1 phase, at the restriction point. Loss of restriction point control is associated with malignant cellular transformation and cancer. For this reason, identifying genes that are differentially expressed in late G1 phase versus quiescence is important for understanding the molecular basis of normal and malignant growth.
Materials and Methods: The differential display (DD) method detects mRNA species that are different between sets of mammalian cells, allowing their recovery and cloning of the corresponding cDNAs. Using this technique, we compared mRNAs from synchronized human breast cancer cells (21PT) in quiescence and in late G1.
Results: Six mRNAs differentially expressed in late G1 or in quiescence were identified. One mRNA expressed 10 hr after serum induction showed 99% homology to a peptide transporter involved in antigen presentation of the class I major histocompatibility complex (TAP-1) mRNA. Another mRNA expressed specifically in quiescence and down-regulated 2 hr following serum induction showed 98% homology to human $NADP^+$-dependent cytoplasmic malic enzyme (EC1.1.1.40) mRNA, which is an important enzyme in fatty acid synthesis and lipogenesis. Three others showed high homology to different mRNAs in the GeneBank, corresponding to genes having unknown functions. Finally, one mRNA revealed no significant homology to known genes in the GeneBank.
Conclusions: We conclude that DD is an efficient and powerful method for the identification of growth-related genes which may have a role in cancer development.

INTRODUCTION

Under the influence of external factors, cells make decisions in late G1 phase of the cell cycle between alternative programs, including going into quiescence (G0) and DNA replication. These decisions are likely to be determined by gene products that are expressed in late G1 phase around the restriction point (1–3). Passage through the restriction point results in loss of requirements for growth factor stimulation and less sensitivity to protein synthesis inhibitors (i.e., cyclohexamide), suggesting that all the protein synthesis required for initiation and progression of these programs are completed in late G1 (1–3). Thus, the late G1 regulatory mechanism most likely controls both the onset of DNA replication and the transcription of mRNAs involved in this process. Loss of restriction point control is found to be associated with malignant transformation and cancer (1,3).

Identification of genes induced or down-regulated in late G1 compared with quiescence (G0)

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should include genes involved in the cellular programs controlled around the restriction point. In order to identify and clone such genes, we first synchronized human breast cancer cells in G0 and in late G1 phase of the cell cycle and then compared the expression of mRNAs by using differential display (DD). This method, originally developed in our laboratory, has now been used successfully for cloning numerous genes (4–8). DD is directed toward the identification of differentially expressed genes, detecting individual mRNA species that are changed in different sets of mammalian cells, and permitting recovery and cloning of their cDNAs (8).

In this study, we synchronized human breast cancer cells (21PT) in late G1 by either using a plant amino acid mimosine, which is a reversible G1 blocker (9,10), or collecting the cells at 8, 10, and 12 hr after serum induction. Quiescence in G0 was maintained by serum starvation. Comparing mRNA expressions in quiescence and in late G1 by using DD, we cloned several differentially expressed mRNAs. Here we report that five corresponding cDNAs showed strong sequence homology to mRNAs from the GeneBank, including TAP-1 (a peptide transporter involved in antigen presentation of class I major histocompability complex [MHC]) (11) and human $NADP^+$-dependent cytoplasmic malic enzyme (an important enzyme in fatty acid synthesis and lipogenesis) (12,13). One cDNA clone had no significant homology to known genes. Possible involvement of identified genes in the growth process and the significance of DD as a method of choice in this study are discussed.

MATERIALS AND METHODS

Cell Culture

The 21PT cell line used in this study, obtained from R. Sager (Harvard Medical School, Boston, MA, U.S.A.) was derived from a human primary breast cancer specimen as described (14). Cells (7.5×10^5) were plated in 150-mm tissue culture plates and grown in α–minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1 μg/ml insulin, 12.5 ng/ml epidermal growth factor, 2.8 μM hydrocortisone, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 mM HEPES buffer, incubated at 37°C in a humidified incubator containing 6.5% CO_2.

Cell Synchronization

For G0 synchronization by serum starvation exponentially growing cultures were washed twice with PBS and placed in α-MEM medium containing 0.4% FBS for 85 hr. For late G1 synchronization, two different methods were used. After 85 hr of serum starvation, cells were either released into α-MEM medium containing 10% FBS with 400 μM mimosine for 24 hr or at Time 0 cells were stimulated by addition of α-MEM medium containing 10% FBS and samples were taken for RNA extraction at 8, 10 and 12 hr. For the analysis of cell cycle–dependent expression of genes, cells synchronized at G0 were stimulated by α-MEM containing 10% FBS, and samples were taken for RNA extraction at indicated times.

Total Cellular RNA Extraction and Northern Blot Analysis

Total cellular RNA extraction was performed with RNAzol-B RNA Extraction Solution (Biotech Lab. Inc., Houston, TX, U.S.A.) according to manufacturer's instructions. Northern blot analyses were performed as described with minor variations (15). Briefly, 20 μg of total RNA from each sample was electrophoresed in 1.1% agarose gel containing 7% formaldehyde, transferred to 0.45-μm nylon membranes (MSI, Westboro, MA, U.S.A.) with 10× SSC, and cross-linked by ultraviolet (UV) irradiation. Prehybridization and hybridization reactions were performed in a microhybridization oven (Belco Glass Inc., Vineland, NJ, U.S.A.) at 42°C using a hybridization buffer containing 50% formamide, 6× SSPE, 5× Denhardt's reagent, 0.1% SDS, and 0.1 mg/ml sheared salmon sperm DNA. Reamplified cDNA probes were purified by 1.5% agarose gel electrophoresis using the QIAEX kit from QIAGEN (Chatsworth, CA, U.S.A.) and labeled with [^{32}P]-dCTP by the random prime labeling kit from Boehringer Mannheim Biochemicals, (Indianapolis, IN) essentially as instructed except 1 μl of 10 μM corresponding oligo dT primer was also included during the labeling. The membranes were washed with 2× SSC, 0.1% SDS for 30 min at room temperature, followed by a wash for 10 min at 55°C in 0.2× SSC, 0.1% SDS. The membranes were then exposed to Kodak X-OMAT AR X-ray films with intensifying screens at −70°C.

Cell Cycle Analysis

Fluorescence-activated cell sorter (FACS) analysis was performed as described (16). Briefly, cells

were trypsinized, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol for 30 min at 4°C, treated with 10 μg/ml RNase for 30 min at 37°C, stained with 70 μM propidium iodide in 38 mM sodium citrate at room temperature for 30 min, and analyzed with a Becton Dickinson cell sorter at the Dana-Farber Cancer Institute FACS analysis facility. CELL-FIT (Becton-Dickinson, Mountain View, CA, U.S.A.) software was used for graphics and statistics.

Differential Display

Differential display (DD) was performed as previously described with minor modifications (8,17). Briefly, 50 μg of total cellular RNA extracted from each sample was treated with DNase I (MessageClean kit; GeneHunter, Brookline, MA, U.S.A.) for removal of chromosomal DNA contamination. Two tenths of a microgram per sample of total RNA from the cells at different time points were reverse transcribed with oligo dT primers and then amplified by polymerase chain reaction (PCR) with arbitrary primers and corresponding oligo dT primers in the presence of 1 μCi/reaction α-[^{33}P]dATP (2000 Ci/mmole; New England Nuclear, Boston, MA, U.S.A.). The following PCR conditions were used: 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for 40 cycles, then 72°C for 5 min. After PCR amplification, the products were resolved by denaturing 6% polyacrylamide gel electrophoresis. After drying the gel and performing autoradiography for screening the bands, differentially expressed cDNAs were cut from the gel and reamplified by PCR with the previous corresponding primer sets. Reamplified cDNAs confirmed by Northern blot analysis were cloned into pCRIII TA cloning vector (Invitrogene, San Diego, CA, U.S.A.), sequenced (Sequenase kit; United States Biochemical Co., Cleveland, OH, U.S.A.) and reprobed for Northern blot analysis after isolation from the plasmid. Differentially expressed cDNA sequences were compared with sequences in GeneBank and EMBL databases via the BLAST network server.

RESULTS

Synchronization of 21PT Cells in Quiescence (G0) and Late G1

The restriction point was defined to be located in late G1, 2 hr before the onset of DNA replication (1). In order to identify and clone differentially expressed genes in late G1 around the restriction point, first we synchronized 21PT cells in quiescence (G0) and in late G1. For this purpose, two sets of experiments were performed and monitored by flow cytometry (Fig. 1 A and B). In the first set of experiments, one population of cells was synchronized in quiescence (G0) by serum starvation and the other population was synchronized in late G1 by restimulation of quiescence cells with complete medium containing 400 μM mimosine for 24 hr. When mimosine was removed and complete medium was added after the mimosine block, cells started DNA synthesis in 4 hr, suggesting that mimosine blocks the cell cycle in late G1 (Fig. 1A). In the second set of experiments, one population of cells was synchronized in quiescence (G0) by serum starvation, as in the first set. However, in this set, instead of a mimosine block, the late G1 population of cells was obtained by restimulating G0 cells with complete medium until they reach late G1 (Fig. 1B). By FACS analysis (Fig. 1C) and thymidine incorporation experiments (data not shown) we calculated that DNA replication in the earliest 21PT cells starts 14 hr after release from quiescence. Thus, taking samples 8, 10, and 12 hr after stimulating cells from quiescence led us catch cells in late G1.

Differentially Expressed mRNAs Identified by Differential Display

After synchronization of 21PT cells at different points in the cell cycle (i.e., G0 versus late G1) total RNAs were extracted from each group as described in Materials and Methods. In order to identify and clone differentially expressed mRNAs in late G1 compared with G0, we used the mRNA differential display method (4,5). After screening through 22 primer combinations with seven different arbitrary and four different oligo dT primers, 25 differentially expressed bands were identified, reamplified, and analyzed by Northern blot analysis. Eight of 25 bands were confirmed to be differentially expressed by Northern blot analysis, another two failed to detect any signals, and the remaining ones were false positives. Six of eight true positive bands were cloned and are reported in this paper. We observed that the bands cut from the DD gel consist of more than one sequence, thus detecting several transcripts on the Northern membrane. In order to obtain single cDNAs, we cloned each band into the pCRIII vector and

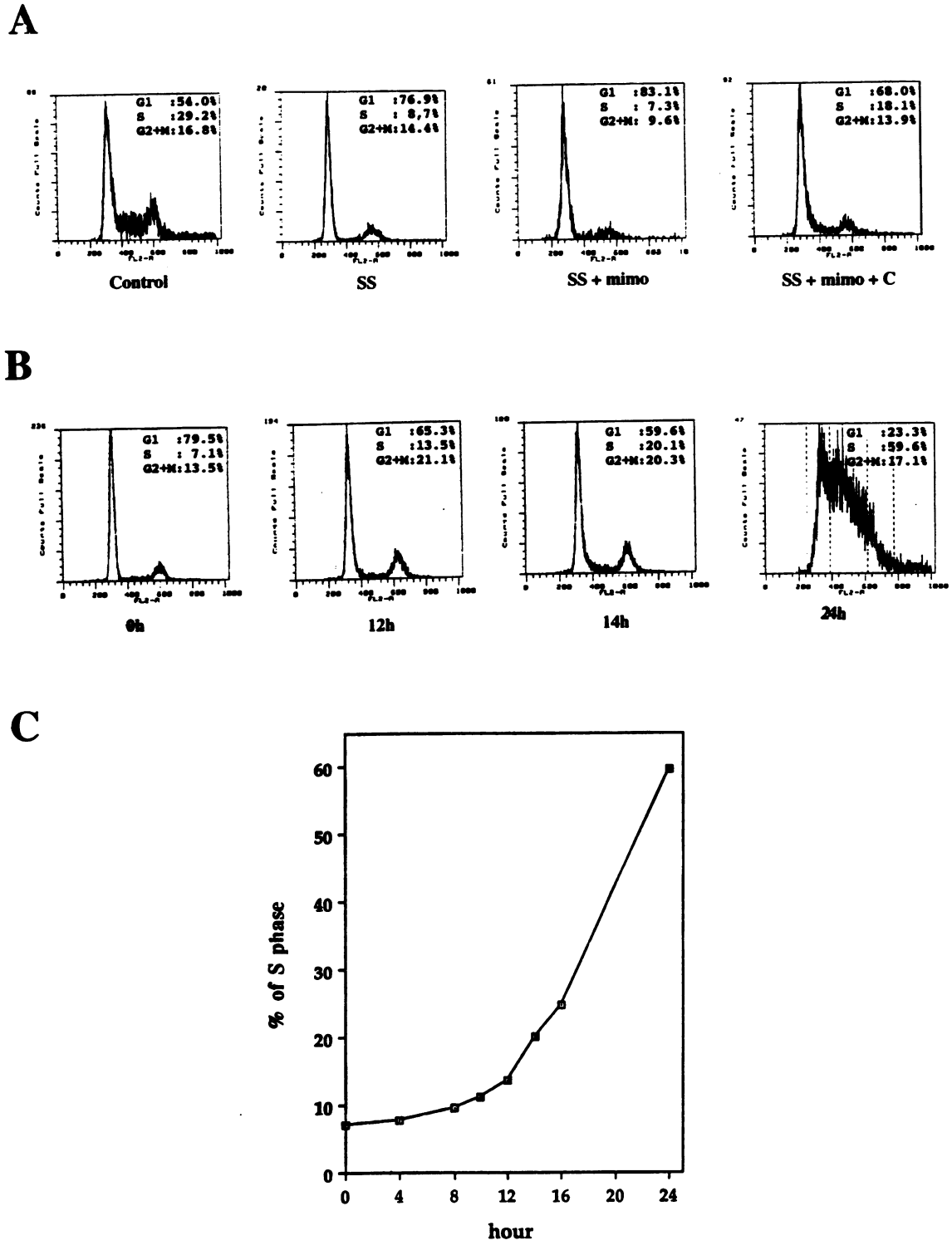

FIG. 1. Synchronization of 21PT cells in G0 and in late G1 with two different approaches

(A) Exponentially growing asynchronous 21PT cells (control) were incubated in serum-deprived medium for 85 hr (SS). After serum starvation, cells were released into α-MEM medium containing 10% FBS with 400 μM mimosine for 24 hr (SS+mimo). Following mimosine block cells were shifted into complete medium without mimosine for 4 hr (SS+mimo+C). (B) Following serum starvation for 85 hr cells were released into complete medium at 0 hr. Samples are shown at 12, 14, and 24 hr after serum induction. (C) Cell cycle progression following serum induction of G0 cells. Samples were taken at indicated time points and monitored by FACS analysis as described in Materials and Methods.

introduced it into competent bacteria (INVαF, Invitrogene). After bacteria was grown in selective media, five colonies were picked and sequenced from each band. Each clone was designated with the number of the band plus a to e. Clones containing different sequences from the same band were probed on Northern membranes, and the one that gave the expected pattern on the Northern was considered a differentially expressed clone. After these confirmations, computer search of GeneBank/EMBL via BLAST network server was performed with these six cDNA sequences (Table 1).

In the first set of experiments, 21PT cells synchronized in G0 by serum starvation, in late G1 by mimosine, and 4 hr after mimosine block release were compared simultaneously by differential display. Three true positive DD bands, designated #1, #34, and #40, were identified and cloned from the first set. Band #1 was detected in the mimosine-treated cells which were blocked in late G1 phase (Fig. 2A). Northern blot analysis with #1 confirmed the DD gel (Fig. 2B). Clone 1a, which was one of the clones from #1 gave the same pattern on the Northern membrane observed with #1, except it lacked the bands at the bottom (Fig. 2C). Sequence analysis of Clone 1a revealed no significant homology with known sequences from the GeneBank. Band #34 was identified in mimosine-treated cells (SS+mimo) and 4 hr after mimosine (SS+mimo+C) (Fig. 2E). Northern confirmations from #34 and Clone 34a gave expected patterns (Fig. 2 F and G). Sequence analysis of Clone 34a revealed 80% homology to a rat gene called SM-20 (18). Band #40 was detected only in G0 cells (Fig. 2H). Differential expressions of #40 and Clone 40b was confirmed by Northern blots (Fig. 2 I and J). Sequence analysis of Clone 40b revealed 98% homology to the 3′ end of human cytoplasmic malic enzyme cDNA (12,13).

In the second set of experiments, 21PT cells were synchronized in G0 as in the first set and samples were taken at 8, 10, and 12 hr after stimulation with complete medium. Three true positive DD bands, named #11, #12, and #15, were identified and cloned from the second set. Northern confirmation of these bands was performed on time course membranes. Band #11 was detected at 8, 10, and 12 hr after stimulation (Fig. 3A). Northern blot analysis of #11 and Clone 11c confirmed the expression pattern observed in the DD gel (Fig. 3 C and D). Sequence analysis of Clone 11c revealed 97% homology to a human cDNA clone with unknown function. Band #12 was detected at 10 and 12 hr after serum stimulation (Fig. 3A). This expression pattern was confirmed with #12 and Clone 12b on Northern blots (Fig. 3 E and F). Sequence analysis of Clone 12b showed 99% homology to 3′ end of human TAP-1 cDNA (a peptide transporter associated with antigen processing). Band #15 was detected at the 10 hr after stimulation (Fig. 3B). Northern blot analysis with #15 and 15d confirmed the expression at 10 hr (Fig. 3 G and H). Sequence analysis of Clone 15d showed 99% homology to a human cDNA (human ORF mRNA) with unknown function. The characteristics of differentially expressed Clones 1a, 34a, 40b, 11c, 12b, and 15d are summarized in Table 1.

Cell Cycle–Dependent Expression of Differentially Expressed Genes

To analyze the cell cycle–dependent expression of identified genes and examine whether the differentially expressed mRNAs identified from the first set are the consequence of specifically expressed mRNAs at late G1 or the consequence of mimosine treatment itself, we made time course membranes from total RNA taken at indicated time points after serum stimulation of G0 cells. Northern blot analysis with time course membranes revealed that five of six genes were expressed in cell cycle–dependent manner. Band #1 and Clone 15d (human ORF mRNA) were detected at 10 hr and disappeared in the following 2 hr (Figs. 2D and 3H). Band #1 was also detected at 24 hr after serum induction (Fig. 2D). Expression of 11c and 12b (TAP-1) was undetectable in quiescent cells, up-regulated in mid and late G1, and continuously expressed in the rest of the cell cycle (Fig. 3 D and F). Clone 40b (cytoplasmic malic enzyme) was expressed in quiescent cells, and its expression was down-regulated after serum stimulation throughout the rest of the cell cycle (Fig. 2K). Although 34a was up-regulated following mimosine treatment (Fig. 2 E–G), we could not detect this up-regulation in cells induced by serum with mimosine-free medium. Thus, we concluded that the up-regulation of 34a in late G1 was not a consequence of serum stimulation but an effect of mimosine itself.

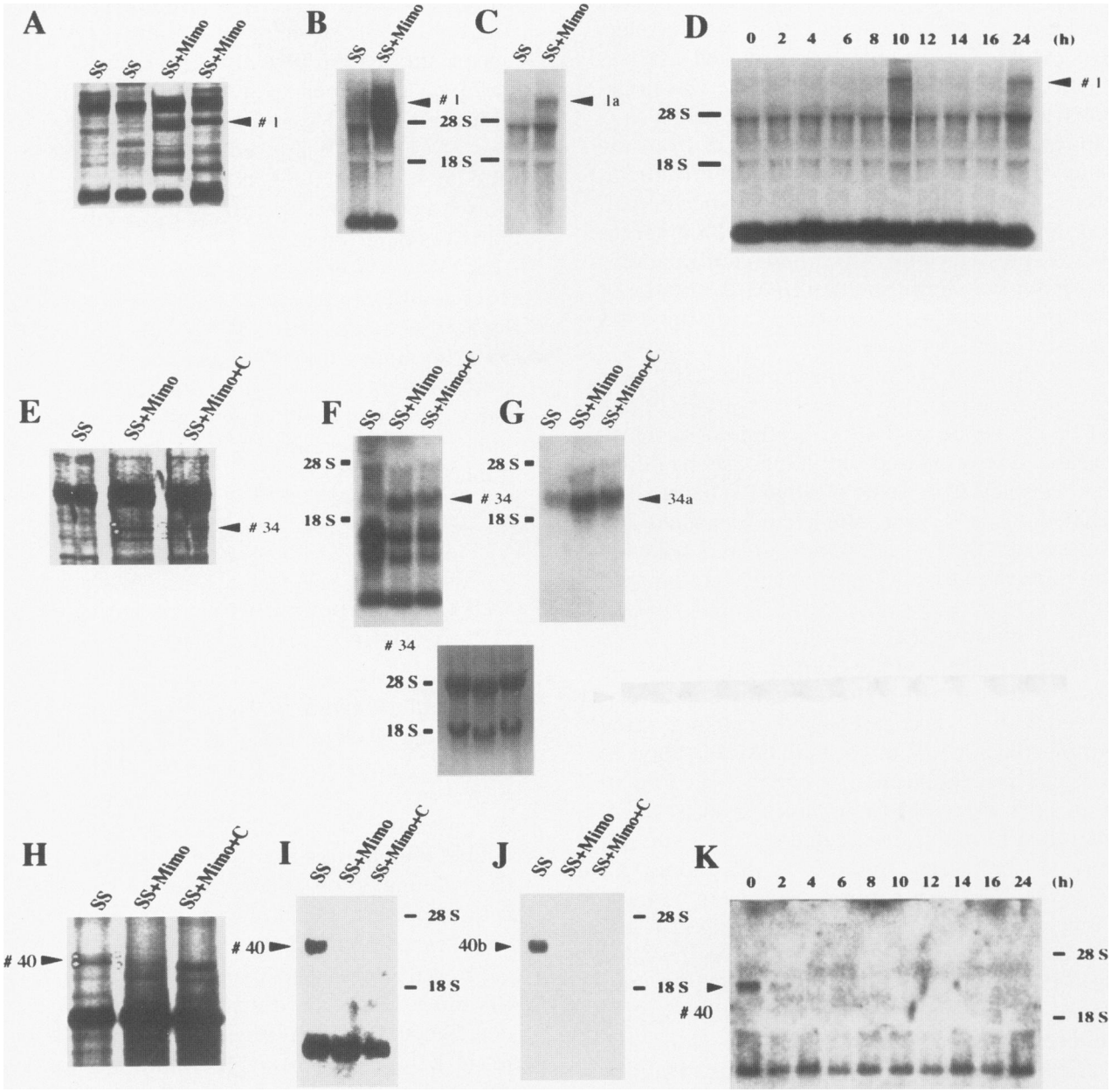

FIG. 2. Identification and expression of cDNA fragments #1, #34, and #40, which are differentially expressed in G0 or in late G1

Mimosine was used for late G1 synchronization (Set 1). Differentially expressed pattern of #1 (A), #34 (E), and #40 (H) on the DD gel. Northern blot confirmation of #1 (B), #34 (F) and #40 (I). After fragments #1, #34, and #40 were cut from DD gel and PCR amplification with the corresponding primers used in DD, Northern blot analysis was performed as described in Materials and Methods. Northern blot analysis of Clones 1a (C), 34a (G), and 40b (J). Clones 1a, 34a, and 40b, each of which was one of the clones from #1, #34, and #40, respectively, were isolated from the plasmids and used as probes for the Northern blot analysis. Expression of #1 (D) and #40 (K) during the cell cycle. Fragment #1 or #40 was labeled and probed on the time course membranes as described in Materials and Methods. SS, 85 hr serum-starved cells; SS+mimo, 85 hr serum starvation + 24 hr 400 μM mimosine treatment in complete medium; SS+mimo+C, 85 hr serum starvation + 24 hr 400 μM mimosine treatment in complete medium + 4 hr complete medium without mimosine.

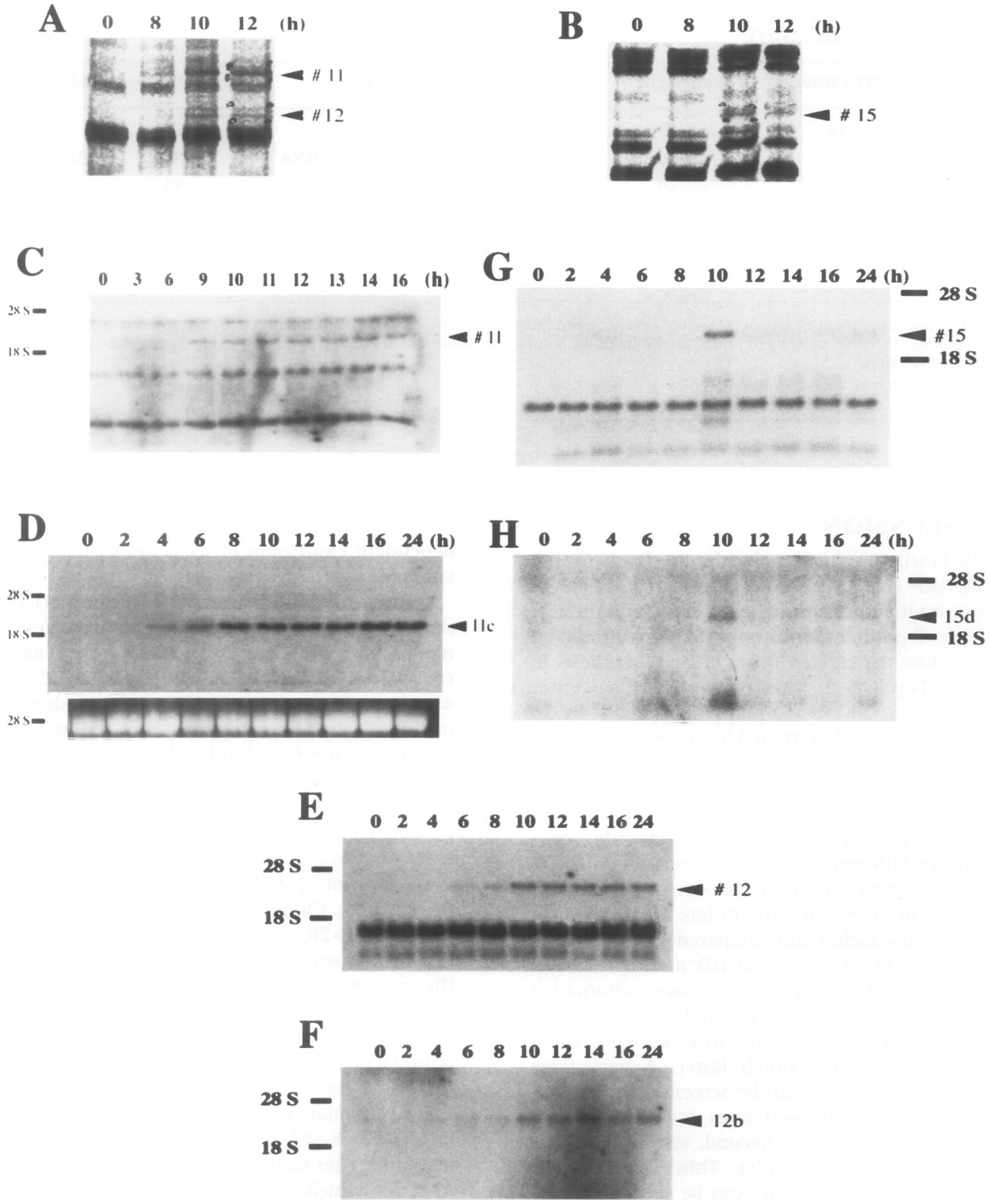

FIG. 3. Identification and expression of cDNA fragments #11, #12, and #15, which are differentially expressed in G0 verse late G1

Time course samples were used to obtain G0 and late G1 cells (Set 2). Differentially expressed patterns of #11 and #12 (A), and #15 (B) on the DD gel. Differential display method was performed on RNA samples collected following serum induction at 0, 8, 10, and 12 hr as described in Materials and Methods. After fragments #11 (C), #12 (E), and #15 (G) were cut from DD gel and PCR amplification with the corresponding primers used in DD, Northern blot analysis was performed with the time course membranes as described in Materials and Methods. Clones 11c (D), 12b (F), and 15d (H), each of which was one of the clones from #11, #12, and #15, respectively, were isolated from the plasmids and used as probes for the Northern blot analysis.

TABLE 1. Characteristics of cDNA clones identified by differential display from human primary breast cancer cells (21PT)

Set[a]	DD Clone	Transcript	Homology	%	Reference[b]
1	1a	8.0 kb	None	—	—
1	34a	3.0 kb	SM-20 rat serum–inducible mRNA	80	U06713
1	40b	3.8 kb	Human malic enzyme	98	X77244
2	11c	2.5 kb	Human partial cDNA	98	Z38301
2	12b	2.8 kb	Human TAP-1	99	X57522
2	15d	2.5 kb	Human ORF cDNA	99	D31885

[a]Set 1, the experiment in which cells were synchronized in G0 by serum starvation and in late G1 by mimosine block; Set 2, the experiment in which cells were synchronized in G0 by serum starvation and in late G1 by catching cells 8, 10, and 12 hr after serum induction.

[b]GeneBank/EMBL accession numbers.

DISCUSSION

The biochemical and molecular mechanisms that regulate cell proliferation are a major field of interest in our laboratory. Our early hypothesis is that cell proliferation is importantly controlled 2 hr before the sudden onset of DNA synthesis in the cell cycle (1,2). The decisions of cells to enter the DNA synthesis (S) phase or to arrest in G0 are likely to be determined by genes expressed in late G1 phase around the restriction point. We proposed that mRNAs differentially expressed in late G1 compared with G0 might be involved in the growth process. In order to identify genes differentially expressed in quiescence versus late G1, we synchronized human breast cancer cells (21PT) in quiescence and in late G1 by two different approaches and compared the mRNA expressions by the powerful DD method. Several technical advantages of DD over alternative methods such as subtractive hybridization made it the method of choice in our study. First, DD is less laborious and much faster. A very large number of mRNAs can be screened and several differentially expressed ones can be identified and cloned in a week. Second, very little RNA is required (0.2 μg/sample). Third, both under- and overexpressed genes can be simultaneously detected on the same DD gel. Fourth, mRNAs can be visualized at each step so that working blindly for about 2 months, as in subtractive hybridization, is eliminated. On the other hand, there are a few technical difficulties with DD, such as false positivity and some DD bands' containing more than one sequence. Here, we report the identification, partial cloning, and cell cycle–dependent expression of six genes by using this strategy.

Three of six differentially expressed cDNAs showed strong homologies to known genes, namely SM-20 (Clone 34a), $NADP^+$-dependent cytoplasmic malic enzyme (EC 1.1.1.40) (Clone 40b), and TAP-1 (Clone 12b). Clone 1a showed no significant homology to any known sequences in the GeneBank. Clones 11c and 15d showed very high homology to cDNA sequences with unknown functions in the GeneBank.

SM-20 was originally identified and cloned from rat vascular smooth muscle cells after the induction of platelet-derived growth factor (PDGF) (18). Clone 34a showed very similar features to SM-20, including 80% sequence identity, which suggested that Clone 34a might be the as yet unknown human homolog of rat SM-20 gene. Although Clone 34a was identified to be up-regulated in mimosine-treated cells, failure to show the same expression pattern on a mimosine-free time course membrane suggested that up-regulation in late G1 is an effect of mimosine itself. SM-20 was shown to be expressed at 30 min after serum induction and disappeared in 2 hr, which represents an immediate early gene (18). The function of this gene is unknown and remains to be determined.

Clone 40b is expressed in quiescent cells and down-regulated in 2 hr after serum induction (Fig. 2 I–K). Sequence analysis of Clone 40b revealed 98% homology to $NADP^+$-dependent cytoplasmic malic enzyme (ME) cDNA, which is

involved in fatty acid synthesis in lipogenesis and catalysis the oxidative decarboxylation of malate into pyruvate (12,13). By this reaction, NADPH is generated from $NADP^+$, which is required for fatty acid synthesis. ME mRNA and protein expression is elevated by triiodothyronine (T3), insulin, and dietary factors such as high carbohydrate, glucose intake, and low fat intake (19). Peroxisome proliferator–activated receptor (PPAR)/retinoid X receptor (RXR) heterodimer, which is important in adipocyte differentiation, transcriptionally activates ME (20). Induction of ME by insulin and T3 is strongly suppressed by EGF (19). The suppressive effect of EGF on ME expression might explain the early inhibition of ME expression after serum induction. Taken together, we showed that ME mRNA expression is significant in quiescent human breast cancer cells and is down-regulated in 2 hr following serum induction. Although there is no evidence about the direct effect of ME on growth regulation, we suggested that some of the upstream regulators of ME such as EGF and PPAR/RXR are involved in the positive and negative regulation of cell growth, respectively.

Clone 12b was not detectable in quiescent cells and was found to be up-regulated in late G1 (Fig. 3F). Computer analysis of clone 12b from the GeneBank showed 99% homology to TAP-1 mRNA, which belongs to the ABC (ATP binding cassette) superfamily of transporters (11). ABC superfamily includes the human multidrug-resistance protein and a series of transporters from bacteria and eucaryotic cells capable of transporting a range of substances, including peptides (11). In one model, TAP-1 protein was proposed to form a heterodimer complex with another ABC superfamily member protein TAP-2 for peptide transporter activity associated with antigen processing necessary for normal assembly of major histocompatibility complex (MHC) class I molecules on the cell membrane (21). Mutation of TAP genes or inhibition of their function by some viral proteins significantly decreases the expression of MHC class I molecules on the cell surface (22,23). Here, we showed that TAP-1 mRNA expression is cell cycle dependent and differentially expressed in proliferating human breast cancer cells. We suggest that immunogenecity of somatic cells might be controlled in part by growth regulation.

#1 and Clone 1a both detected at least three transcripts, of which only one was differentially expressed (Fig. 2 B–D). The differentially expressed transcript was about 8 kb and was expressed at 10 and 24 hr after serum induction (Fig. 2D). Sequence analysis of Clone 1a revealed no significant homology with known sequences. Expression timing coincidentally overlaps with late G1 and late S phase of the cell cycle, suggesting a cyclin E or A type expression pattern. Clones 11c and 15d showed very strong homology to human cDNAs with unknown function in the GeneBank. Clone 11c was detected in mid G1 and in the rest of cell cycle (Fig. 3D). Clone 15d detected a transcript expressed only at 10 hr after serum induction (Fig. 3H). Coincident expression of #1 and Clone 15d at 10 hr after serum induction supports the significance of late G1 following serum stimulation.

In conclusion, we identified six cDNAs differentially expressed genes in late G1 or in quiescence by using the DD method and showed the cell cycle–dependent expression patterns of these genes. We conclude that DD is an efficient and powerful method for the identifying of known and unknown growth-related genes as well as many other genes with different functions.

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