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Transcriptional Regulation of the Human CAD Gene during Myeloid Differentiation

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CAD codes for a trifunctional protein involved in the catalysis of the first three enzymatic activities in the de novo pyrimidine biosynthetic pathway, namely, carbamoyl-phosphate synthetase II (EC 6.3.5.5), aspartate transcarbamylase (EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3). CAD regulation was studied in the human promyelocyte leukemic line HL-60 as it differentiated into monocytic or granulocytic lineages after induction by 12-*O*-tetradecanoylphorbol-13-acetate or *trans*-retinoic acid and dibutyryl cyclic AMP, respectively. Within 12 h of induction of HL-60 cells with either inducer, total cellular levels of CAD RNA essentially disappeared. On the other hand, no apparent decreases in β-actin RNA levels were seen even 48 h after HL-60 cells were induced, as compared with untreated cells. With nuclear runoff assays, it was clearly shown that the inactivation of CAD gene expression during the induction of HL-60 cells with either inducer was at the transcriptional level. The nuclear runoff experiments also demonstrated that the CAD gene expression was shut down in less than 4 h after induction, well before morphological changes were observed in these cells. At the enzymatic level, the activity of aspartate transcarbamylase, one of the three enzymes encoded by the CAD gene, decreased by about half within 24 h of induction, suggesting a CAD protein half-life of 24 h in differentiating HL-60 cells. Nevertheless, this means that significant levels of aspartate transcarbamylase activity remained even after the cells have stopped proliferating. From the RNA data, it is clear that CAD gene expression is rapidly turned off as promyelocytes begin to terminally differentiate into macrophages and granulocytes. We suspect that the inactivation of the CAD gene in induced HL-60 cells is a consequence of the differentiating cells leaving the cell cycle and becoming nonproliferating.

The de novo pyrimidne biosynthetic pathway provides pyrimidines for nucleic acid synthesis. Pyrimidine biosynthesis is catalyzed by six enzymatic activities: carbamoyl-phosphate synthetase II (EC 6.3.5.5), aspartate transcarbamylase (EC 2.1.3.2), dihydroorotase (EC 3.5.2.3), dihydroorotate dehydrogenase (EC 1.3.99.11), orotate phosphoribosyltransferase (EC 2.4.2.10), and orotidylate decarboxylase (EC 4.1.1.23) (19, 36). In mammals, the first three and the last two enzymatic activities of the pathway are associated with the multifunctional proteins, CAD and UMP synthase, respectively (9, 19, 36). Elevated activities of the de novo pyrimidine biosynthetic enzymes have been reported in the early stages of fetal development (15, 23, 31, 35) and in especially rapidly growing cells and tissues, such as ascites tumor cells (5), hepatoma nodules (6), regenerating liver (6), and a variety of other tumor cells (21). Although the regulation of de novo pyrimidine biosynthesis in actively proliferating cells has been a subject of intensive investigation for the last 10 years, it has been confined to studying levels of enzymatic activities (7, 23, 36). Hitherto, there have been no reports on the regulation of genes encoding enzymes of the pyrimidine biosynthetic pathway. Since pyrimidine biosynthetic activity is severalfold higher in actively proliferating cells than in slower growing or nondividing cells, we propose that expression of the genes coding for the enzymes of the de novo pyrimidine biosynthesis is cell growth dependent and is restricted to part of the cell cycle. To obtain evidence in support of this hypothesis, we have chosen to study the HL-60 cell system. HL-60 is a cell line derived from a human promyelocytic leukemia (10, 11). This is a powerful model system for both cellular proliferation and differentiation. Proliferating HL-60 cells can be stimulated to terminally differentiate into either nonproliferating monocytic (30) or granulocytic (4, 14) lineages by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or with a combination of retinoic acid (RA) and dibutyryl cyclic AMP (cAMP), respectively. Using this system, we have examined the regulation of CAD gene expression. We show here that CAD is regulated at the transcription level and its expression appears to be linked to the proliferative state of the cell.

Cells. HL-60 and HT-1080 fibrosarcoma cells were obtained from the American Type Culture Collection, Rockville, Md. The HL-60 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO Laboratories), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.0), and 40 μg of gentamicin per ml. Cultures were maintained in humidified 95% air–5% CO_2 at 37°C by passage of 2×10^5 to 5×10^5 cells per ml every 3 days. To induce cells with TPA or RA and cAMP, we washed and suspended the cells in fresh RPMI 1640 medium containing either TPA (20 ng/ml) or RA (0.3 μg/ml) and cAMP (0.5 mg/ml). Since TPA was dissolved in dimethyl sulfoxide (Me_2SO), control cultures were grown in 0.002% Me_2SO (vol/vol), which was equivalent to the concentration of Me_2SO in TPA-induced cultures. HT-1080 cells were grown in Ham F12 medium supplemented with 10% (vol/vol) fetal bovine serum.

Cell viability was tested by the Trypan blue exclusion assay. The viability of induced HL-60 cells was further tested by [^{14}C]leucine incorporation into trichloroacetic acid-precipitable proteins. In this assay, HL-60 cells with and without 24 h of TPA induction were cultured for 2 h in medium containing 1 μCi of [^{14}C]leucine per ml (specific

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activity, 270 mCi/mmol), 10 μM unlabeled leucine, and 10% (vol/vol) dialyzed fetal bovine serum. Cells were washed thoroughly with phosphate-buffered saline and lysed by sonication by the procedures described previously (13). To 100-μl samples of cell lysate, 100 μg of bovine serum albumin was added. Then an equal volume of cold 10% trichloroacetic acid was added, and the protein was precipitated by incubating the mixture on ice for 10 min. The precipitate was recovered by passage through a 25-mm Whatman GF/A glass microfiber filter. The filter was washed with 10 ml of ice-cold 5% trichloroacetic acid and air dried. After drying, the filter was placed in a liquid scintillation vial containing 3 ml of liquid scintillant fluid. The radioactivity was counted in a Beckman LS9000 liquid scintillation counter.

Isolation and analysis of total cellular RNA. Cells were harvested by centrifugation and washed with phosphate-buffered saline. Total cellular RNA was isolated essentially by the guanidine isothiocyanate-cesium chloride protocol of Chirgwin et al. (8). The RNA was glyoxalated, electrophoresed on 1% agarose gels (40 μg of RNA per lane), and transferred to BA85 nitrocellulose filters (Schleicher & Schuell) by the method of Thomas (37). These filters were hybridized with the nick-translated plasmid pHA21 which carried a unique-sequence (1,950-base-pair) genomic fragment of human CAD gene coding for a portion of the aspartate transcarbamylase domain of the CAD protein (details about this probe will be published elsewhere). Nick translation was carried out with a Bethesda Research Laboratories nick translation kit essentially per their protocol with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) (New England Nuclear Corp.) as a labeled nucleotide. Prehybridization, hybridization, and filter washing were done as described by Meinkoth and Wahl (29). Filters were exposed to Kodak XAR X-ray film with intensifying screens at −70°C for 1 to 3 days. Filters were probed with human β-actin probe (pHF5) (17) after the first probe (CAD) was removed by washing the filters at 100°C for 1 h in water. Quantitation of bands on autoradiograms was done with a densitometer (E-C Apparatus Corp.).

Nuclear RNA runoff assays. Nuclei from induced and uninduced HL-60 cells were isolated by the technique of Groudine et al. (16). Transcription in isolated nuclei and RNA purification were done by the method of Linial et al. (27). Briefly, the reaction mixture consisting of 210 μl of nuclei (3×10^7 to 5×10^7 nuclei per assay), 60 μl of nuclear runoff buffer (25 mM Tris hydrochloride [pH 8.0], 12.5 mM $MgCl_2$, 750 mM KCl, and 1.25 mM each ATP, GTP, and CTP), and 50 μl of [α-^{32}P]UTP (specific activity, 3,000 Ci/mmol) (New England Nuclear) was incubated at 30°C for 30 min. For control experiments, α-amanitin, a potent inhibitor of RNA polymerase II, was added to the reaction mixture at a concentration of 1 μg/ml. Reactions were terminated by the addition of 8 μl of 11-mg/ml RNase-free DNase I and by a 5-min incubation at 30°C. The labeled RNA was purified by the procedure of Groudine et al. (16). The labeled RNA pellet was suspended in hybridization buffer {100 mM Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] [pH 7.4], 0.2% sodium dodecyl sulfate, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt solution, and 250 μg of *Escherichia coli* RNA per ml} and used as a probe. Plasmid DNAs carrying CAD and β-actin genes were linearized by restriction endonuclease digestion. Linearized plasmid DNAs were denatured by incubation with a final concentration of 0.2 M NaOH for 30 min at room temperature. After denaturation, the DNA solutions were neutralized by the addition of 10 volumes of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and applied onto BA85 nitrocellulose filters (10 μg of DNA per slot) by using a Schleicher & Schuell slot blot apparatus and gentle suction. The filters were prehybridized in 3.0 ml of hybridization buffer for 3 h at 65°C. After prehybridization, filters were hybridized in 2.0 ml of hybridization buffer containing ^{32}P-labeled nuclear RNA transcripts at a concentration of 10^7 cpM/ml for 48 h at 65°C. The filters were then washed twice for 15 min in 0.1% sodium dodecyl sulfate–2× SSC at room temperature and for 60 min in 0.1% sodium dodecyl sulfate–0.1× SSC at 60°C. Filters were dried and exposed to Kodak XAR X-ray film with an intensifying screen at −70°C for 1 to 3 days.

TABLE 1. Percentage of HL-60 cells differentiating into monocytes after TPA induction

Induction time (h)	% Cells in suspension	% Cells adherent
0	100	0
4	96	4
24	65	35
48	33	67
72	30	70

Aspartate transcarbamylase assay. Aspartate transcarbamylase activity in induced and uninduced HL-60 cells was measured by the method of Shoaf and Jones (36) as described previously (33) with [^{14}C]aspartate (specific activity, 231 mCi/mmol) (New England Nuclear) as one of its substrates. Enzyme activity was expressed as nanomoles of *N*-carbamylaspartate formed per milligram of protein per minute. Protein content was determined by the method of Bradford (3).

Induction of HL-60 cells. HL-60 cells were induced to differentiate into macrophages by treatment with TPA. Morphological maturation of macrophages was assessed by microscopic examination. In contrast to untreated HL-60 cells, which are typically round and grow as single cells in suspension, TPA-treated cells became adherent and took on the appearance of macrophages. These morphological changes were seen in about 4% of cells by 4 h after TPA treatment. By 48 h after TPA induction, nearly 70% of the cells became adherent (Table 1). Cell viability, as tested by Trypan blue exclusion, indicated that more than 90% of the adherent cells were viable 48 h after TPA induction. When [^{14}C]leucine incorporation into trichloroacetic acid-precipitable counts was used as an indication of protein synthesis, adherent cells (induced with TPA for 24 h) showed 60% of the protein synthesis capacity of the nonadherent, uninduced cells.

In the second system, HL-60 cells were induced with a combination of RA and cAMP. RA alone induces HL-60 cells to differentiate into granulocytes (4). We confirmed the observation of Davies et al. [14] that addition of cAMP enhances the effect of RA on the induction of the differentiation of HL-60 cells into granulocytes. Because the cells remain in suspension in this system, the morphological maturation of granulocytes was assessed by the appearance of nuclear pleomorphism and loss of nucleoli. By 48 h after induction, nearly 10% of cells were fully differentiated, and their viability was about 90%. Once cells were induced by either means, cells ceased proliferation, as is evident from the results on cell growth (Fig. 1).

Analysis of CAD RNA levels in induced and uninduced HL-60 cells. Northern blot analysis of total cellular RNA

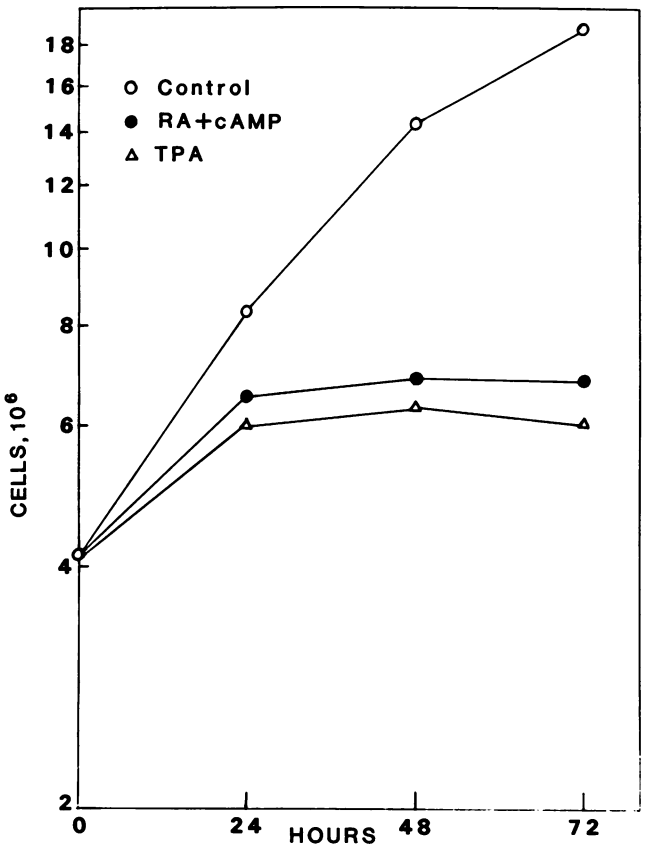

FIG. 1. Effect of inducers on growth rate of HL-60 cells. Cells were plated at a concentration of 5.17×10^5/ml of medium and were cultured for various time intervals in the presence of inducers. An inducer was not present in the control. After an appropriate time period, the cells were harvested, and the total cell number was counted with a hemacytometer.

isolated after 0, 12, 24, and 48 h of TPA induction clearly showed that CAD RNA had essentially disappeared even after only 12 h of induction (Fig. 2A). To determine the point after induction at which CAD RNA starts to decline, total cellular RNA was isolated from cells after 4 and 8 h of induction. Cellular levels of CAD RNA must begin to decline sometime between 4 and 8 h after induction (Fig. 2C). A similar pattern of results was obtained when HL-60 cells were induced instead with a combination of RA and cAMP (Fig. 2B and D). On the other hand, no apparent decreases in actin RNA levels were observed in HL-60 cells induced with either inducer, as compared to uninduced cells (Fig. 2). In fact, in some experiments actin RNA levels appeared to increase after induction, an observation similar to that of Bentley and Groudine (2). These experiments imply that, while actin RNA levels are essentially unaffected by inducing myeloid differentiation in HL-60 cells, CAD RNA vanishes with differentiation. Furthermore, the high levels of actin RNA so long after induction are another indication that the HL-60 cells are generally healthy and that the disappearance of CAD RNA is in no way a reflection of dying cells.

There is always the possibility that the decrease in CAD RNA levels is not due to a regulated shutdown of gene expression but rather to a direct interaction with the inducers used. To obtain evidence against this latter possibility, we did experiments with HT-1080 cells. The inducer TPA has no effect on the growth of HT-1080 cells. Hence, unless TPA has a direct effect on CAD expression, levels of CAD RNA in these cells should remain unchanged whether or not the cells are treated with TPA. In fact, treating the HT-1080 cells for 24 h with TPA had no effect on CAD RNA levels (data not shown).

Nuclear RNA runoff analysis. To ascertain whether the decrease in CAD RNA in induced HL-60 cells was a consequence of transcriptional inactivation of the CAD gene or posttranscriptional regulation (e.g., altered stability of CAD primary transcripts), we isolated nuclei from both induced and uninduced HL-60 cells. Nascent RNA was labeled by chain elongation in the presence of [α-^{32}P]UTP. The RNA transcripts initiated in vivo and elongated in vitro were hybridized to CAD DNA immobilized on nitrocellulose filters. Significant hybridization to the CAD DNA was observed with labeled nuclear RNA transcripts isolated from uninduced HL-60 cells (Fig. 3). However, even after only 4 h of induction with either TPA or RA and cAMP, little to no hybridization to CAD DNA was observed (Fig. 3). At the

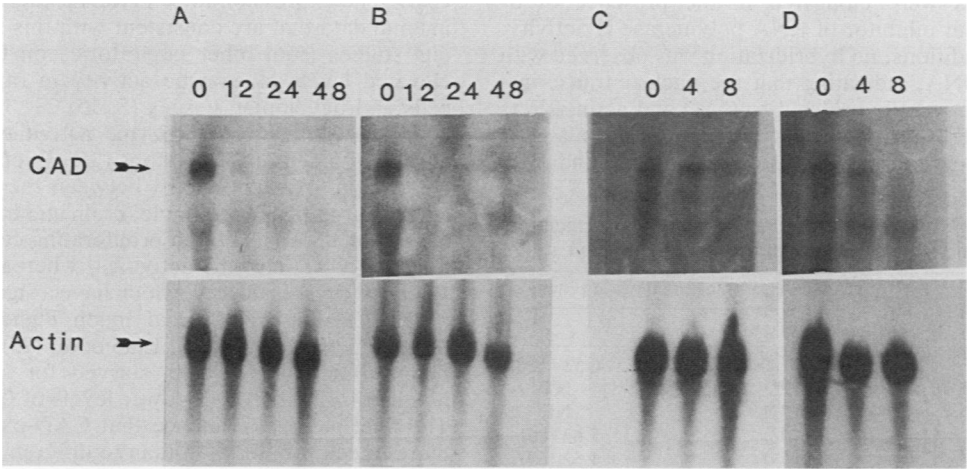

FIG. 2. Northern blot analysis of CAD and actin RNA. (A) Total cellular RNA from 0-, 12-, 24-, and 48-h TPA-induced HL-60 cells was electrophoresed on 1% agarose gels (40 μg per lane), transferred onto nitrocellulose, and probed with nick-translated pHA21 and pHF5 DNA, as described in the text. (B) Same as in panel A but the cells were treated with a combination of RA and cAMP. (C) Same as in panel A but the cells were induced with TPA for 0, 4, and 8 h, respectively. (D) Same as in panel C but the cells were treated with a combination of RA and cAMP.

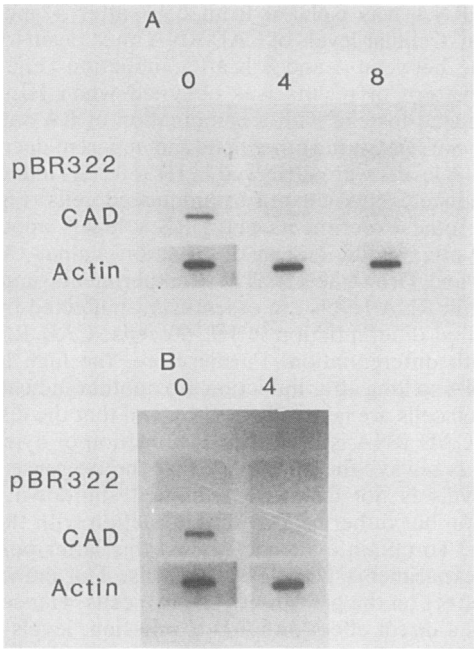

FIG. 3. (A) Hybridization of in vitro synthesis nuclear [^{32}P]RNA to CAD, actin or pBR322 DNA. Nuclei were prepared from 0-, 4-, and 8-h TPA-induced HL-60 cells. Nuclear transcription was carried out as described in the text. ^{32}P-labeled nuclear RNAs thus isolated were hybridized to CAD, actin, and pBR322 DNA immobilized on nitrocellulose (10 μg per slot). (B) Same as in panel A except that the nuclei were prepared from 0- and 4-h RA- and cAMP-treated HL-60 cells.

same time, no significant differences in actin nuclear transcription were observed between induced and uninduced HL-60 cells (Fig. 3). This indicates that, while actin transcription continues during myeloid differentiation, CAD transcription becomes inactivated in the induced cells. The results also revealed that the transcriptional inactivation of CAD is an early event in the terminal differentiation of macrophages and granulocytes.

Two control experiments were also performed. Nuclear RNA transcripts were elongated in the presence of α-amanitin, a potent inhibitor of RNA polymerase II activity. Under these conditions, no hybridization was observed with CAD or actin DNA, indicating that the nuclear transcripts elongated in vitro and hybridized to CAD and actin DNAs are RNA polymerase II dependent. Then, too, when labeled nuclear transcripts were hybridized to filters bound with pBR322 DNA containing no CAD or actin insert, no hybridization was observed, a result which indicated that the hybridizations seen with the CAD and actin DNAs were specific.

TABLE 2. Aspartate transcarbamylase activity during induction of HL-60 monocytic and granulocytic differentiation

Induction time (h)	Aspartate transcarbamylase activity[a]	
	TPA	RA + cAMP
0	6.53 (100)	6.53 (100)
4	4.63 (71)	ND
8	4.78 (73)	ND
12	5.33 (82)	5.62 (86)
24	3.46 (53)	4.52 (69)
48	1.76 (27)	2.35 (36)

[a] Aspartate transcarbamylase activity is expressed as nanomoles of *N*-carbamylaspartate formed per milligram of protein per minute. Numbers in parentheses indicate the percentages of control activity remaining. ND, Not determined.

Aspartate transcarbamylase activity. Aspartate transcarbamylase activity in induced and uninduced HL-60 cells is shown in Table 2. During the first 12 h after cells were induced to differentiate, aspartate transcarbamylase activity did not change significantly from that of uninduced cells (Table 2). At time points after 12 h of induction with either inducer, aspartate transcarbamylase activity showed a steady decrease (Table 2). Eventually, the enzyme activity was decreasing at a rate of about 50% per 24 h.

In recent years, many investigators have been studying the expression of genes encoding enzymes of metabolic pathways, such as dihydrofolate reductase (1, 18, 22, 28) and thymidylate synthetase (12, 20), to understand the mechanism of their regulation during cell growth. As the expression of these genes in normal cells is very low, the above studies have focused mainly, if not exclusively, on cell lines amplified for these genes. These studies have shown that the regulation of the expression of these genes is cell growth dependent. Depending upon the system studied, the same gene appears to be regulated sometimes at the transcriptional level (34) and sometimes at the posttranscriptional level (24). In our studies, we selected the HL-60 cell system. HL-60 is a well-characterized human cell line with the potential to differentiate into nonproliferating monocytes (26, 30) or granulocytes (4, 14). Furthermore, this system can mimic normal myeloid development (4). Using this system, we have shown that the expression of the CAD gene, a gene coding for a trifunctional protein involved in pyrimidine biosynthesis, is regulated during the transition of a proliferative cell into a nondividing differentiated cell. Induction of HL-60 cells to differentiate along either a monocytic or granulocytic pathway is accompanied by a nearly complete disappearance in the steady-state levels and transcription of CAD RNA.

Such changes in CAD expression reflect either the fact that the HL60 cells are terminally differentiating or the fact that the induced cells become nonproliferating. We believe that the latter is correct. While further work with this and other systems will be necessary to support the linkage of CAD gene expression to cell growth, there is already experimental data that are consistent with this hypothesis. Previous studies from other laboratories on CAD have shown elevated levels of enzyme activity in rapidly proliferating cells and developing tissues (5, 15, 23, 31, 35). It also has been shown that CAD enzyme activities decrease as the growth of a cell or tissue stops (35). Even in adult tissues a correlation appears to exist between the enzymes of CAD and cell growth. For example, brain and heart tissues, which have a small proportion of proliferating cells, have very low levels of CAD enzyme activities, whereas tissues, such as spleen or thymus tissues, which have a significant proportion of proliferating cells, have much higher levels of CAD enzyme activities (31, 35). Liao et al. (25) recently reported that Syrian hamster cells starved for serum, and hence nondividing, had 10-fold-lower levels of CAD transcription. The final piece of evidence that CAD expression is linked more to cell proliferation than to differentiation comes from work described here. While CAD RNA has essentially disappeared 12 h after induction with TPA or RA and cAMP, most of the cells (90% in the case of granulocyte development) showed few signs typical of mature macrophages or granulocytes. Hence, the down regulation of CAD expres-

sion is a relatively early event during the induced differentiation of HL-60 cells.

The nuclear RNA runoff experiments showed that the decreases in the steady-state levels of CAD RNA during induction of HL-60 cell differentiation are due to the inactivation of the CAD gene at the transcriptional level, not at the posttranscriptional level. Because only a 3′ probe was used to examine CAD transcription, it is not possible to ascertain where the block of transcription developed. Regulation at the elongation step of transcription has recently been demonstrated in the case of the growth-dependent c-*myc* gene (2). The significant decline in the steady-state levels of CAD RNA 8 h after induction and the transcriptional inactivation of the CAD gene within 4 h after induction together suggest a half-life of less than 4 h for CAD RNA in induced cells. The half-life of CAD RNA in uninduced HL-60 cells is yet undetermined.

The changes in CAD expression accompanying the differentiation of HL-60 cells may extend to the protein level as well. The activity of aspartate transcarbamylase, one of three enzymes encoded by the CAD gene, decreases at an ever increasing rate after induction with either inducer, eventually reaching a rate approaching a 50% decline per 24 h in induced cells. Padgett et al. (32) have reported a half-life of about 75 h for CAD protein in exponentially growing Syrian hamster cells. If the half-life of CAD protein in uninduced HL-60 cells is the same as that in exponentially growing Syrian hamster cells, then the higher-than-expected decrease in aspartate transcarbamylase activity in induced HL-60 cells might be explained by either an increased rate of degradation of CAD protein or some form of inactivation of the aspartate transcarbamylase activity. Regardless which explanation is correct, this suggests that induction of HL-60 cell differentiation may lead to posttranslational regulation of CAD. Additional experiments are necessary to determine the half-life of CAD protein in uninduced HL-60 cells.

At 24 h after induction, when the HL-60 cells are clearly no longer proliferating and the CAD gene is transcriptionally inactive, there is still over 50% of the aspartate transcarbamylase activity, as compared to uninduced cells. If the other five enzymes of de novo pyrimidine biosynthesis are similarly affected, then it is unlikely that the nonproliferation of induced HL-60 cells is due to a shortage of pyrimidines. Previous experiments with Chinese hamster cell mutants defective in CAD support this assertion. We have observed that such mutants are capable of growing in the absence of exogenous pyrimidines when the activities of the first three enzymes of pyrimidine biosynthesis are below 20% of the wild-type levels (13). Hence, we believe that the induced HL-60 cells stop proliferating as a result of factors other than cellular levels of pyrimidines.

Why then is CAD gene expression inactivated when HL-60 cells are induced with TPA or RA and cAMP? All available data about the CAD gene and the enzyme activities associated with the CAD protein make sense if the CAD gene is actively transcribed during a restricted portion of the cell cycle (e.g., late G_1 or early S). Hence, in rapidly growing cells, the enzyme activities associated with CAD would be at high levels due to CAD synthesis in each cell cycle and the long half-life of the protein. On the other hand, in differentiating cells (such as induced HL-60 cells) that leave the cell cycle, the CAD gene will not be expressed, and the enzyme activities associated with CAD would decline over time as the protein is turned over and not replaced by new synthesis. Further experiments are needed to demonstrate that CAD gene expression is cell cycle dependent, thus explaining the relationship between the levels of enzyme activities of CAD and rates of cellular proliferation.

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