Volume 232, number 1, 238–242 FEB 05865 May 1988

Posttranscriptional regulation of the expression of CAD gene during differentiation of F9 teratocarcinoma cells by induction with retinoic acid and dibutyryl cyclic AMP

Gadiparthi N. Rao*+, Robert L. Church* and Jeffrey N. Davidson+

Department of Ophthalmology, Emory University School of Medicine, 1327 Clifton Rd NE, Atlanta, GA 30322 and +*Department of Microbiology and Immunology, University of Kentucky Medical Center, Lexington, KY 40536, USA*

Received 1 February 1988; revised version received 24 March 1988

We have studied the regulation of expression of the carbamoyl-phosphate synthetase II-aspartate transcarbamylase-dihydroorotase gene in F9 teratocarcinoma cells during their differentiation into parietal endoderm cells by induction with a combination of retinoic acid and dibutyryl cyclic AMP. Steady-state levels of CAD mRNA decreased by 7-fold in F9 cells following 120 h of retinoic acid and dibutyryl cyclic AMP induction as compared to levels in uninduced cells. Conversely, no apparent changes were found in the steady-state levels of β-actin mRNA between induced and uninduced cells. Despite a 7-fold decrease in the steady-state levels of CAD mRNA, its rate of transcription remained the same between induced and uninduced cells, indicating a role for posttranscriptional mechanisms for its down regulation during retinoic acid- and dibutyryl cyclic AMP-induced differentiation of F9 cells. The cellular growth rate of F9 cells as determined by [^{3}H]thymidine uptake and parallel cell counting decreased markedly during their induction with retinoic acid and dibutyryl cyclic AMP. Taken together, it is apparent that the expression of the CAD gene is cell-growth-dependent and its regulation in this system is at the posttranscriptional level.

CAD gene; mRNA; Enzyme activity; Aspartate transcarbamylase; Cell growth; Differentiation; Pyrimidine

1. INTRODUCTION

The de novo pyrimidine biosynthetic pathway plays a pivotal role in the supply of pyrimidines for nucleic acid biosynthesis. This pathway consists of six enzymatic steps, namely 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), orotidylate phosphoribosyltransferase (EC 2.4.2.10) and orotidylate decarboxylase (EC 4.1.1.23) [1]. Fetal tissues have been reported to have higher activities of these enzymes than the corresponding adult tissues [2–5]. Elevated activities of the enzymes of de novo pyrimidine biosynthesis have also been reported in a variety of tumor cells such as Ehrlich ascites [6], hepatoma [7] and sarcoma [7]. While these studies revealed a positive correlation between the activities of the enzymes of de novo pyrimidine biosynthesis and cellular growth, the mechanism of its developmental regulation is yet to be defined. Based on isolated enzymatic and $^{14}CO_2$ incorporation studies, it was found that the de novo pyrimidine biosynthetic pathway is regulated by feedback control [8–10]. Even then, it remains unclear particularly with respect to the genomic regulation of de novo pyrimidine biosynthesis during cellular growth. To this end, we have made an attempt to study the regulation of expression of the CAD gene, which codes for a trifunctional protein involved in the catalysis of the first three enzymatic activities of de novo pyrimidine biosynthesis, namely carbamoyl-phosphate synthetase II (CPSase), aspartate transcarbamylase (ATCase) and dihydroorotase (DHOase) [11], in F9

Correspondence address: G.N. Rao, Department of Ophthalmology, Emory University School of Medicine, 1327 Clifton Road NE, Atlanta, GA 30322, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

teratocarcinoma cells during retinoic acid (RA) and dibutyryl cyclic AMP (cAMP) induced differentiation. These cells, upon induction with RA and cAMP, cease to proliferate and instead differentiate into parietal endoderm cells [12–14]. Hence, these cells provide a better model system for studying the biological activities associated with cellular growth. Here, we report that CAD mRNA is reduced substantially during cellular growth arrest and its down regulation in this system is at the posttranscriptional level.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Mouse F9 teratocarcinoma cells were provided by Dr Betsy Moore of the University of Colorado Health Sciences Center (Denver, CO). These cells were grown in Ham's F12 medium supplemented with 15% (v/v) fetal bovine serum. Cultures were maintained in humidified 95% air-5% CO_2 at 37°C and passaged at 3–5 × 10^5 cells/ml every 3 days. For induction, cells were grown for 1 day without inducers in the medium and the next day the medium was replaced by fresh medium containing inducers, RA (0.3 μg/ml) and cAMP (0.5 mg/ml). Cell viability was tested as in [15].

2.2. *RNA isolation*

Total cellular RNA was isolated from induced and uninduced F9 cells by the guanidine isothiocyanate-cesium chloride protocol of Chirgwin et al. [16] as described in [15]. Equal amounts of total cellular RNA from both induced and uninduced F9 cells were size fractionated on 1% agarose containing 6.6% formaldehyde and then transferred to BA85 nitrocellulose filters according to Thomas [17]. The filters were baked for 2 h at 80°C under vacuum and subsequently hybridized to nick-translated probes. Nick-translation was carried out with a BRL nick-translation kit using [α-^{32}P]dCTP (spec. act. 3000 Ci/mmol, NEN) essentially as per the supplier's directions. Details of the pCAD142 probe have been published elsewhere [11]. β-Actin probe (pHF5) [18] was provided by Dr Jerry Woodward of the University of Kentucky Medical Center (Lexington, KY). Prehybridization, hybridization and washing conditions for the filters were the same as those described by Meinkoth and Wahl [19].

2.3. *Transcription assay*

Nuclei from both induced and uninduced F9 cells were isolated according to Groudine et al. [20]. RNA labeling in isolated nuclei using [α-^{32}P]UTP (spec. act. 3000 Ci/mmol, NEN) and its further isolation and purification were performed as described by Linial et al. [21]. Hybridization of labeled RNA transcripts to the gene DNAs immobilized on BA85 nitrocellulose filters and subsequent washings were performed according to Linial et al. [21].

2.4. *ATCase assay*

ATCase activity in induced and uninduced F9 cells was measured by the method of Shoaf and Jones [1] using [U-^{14}C]aspartate (spec. act. 231 mCi/mmol, NEN) as one of its substrates. Enzyme activity is expressed as nmol *N*-carbamoyl L-aspartate formed/mg protein per min. Protein content was determined according to Bradford [22].

2.5. *DNA synthesis*

Cells with and without an appropriate period of induction with RA and cAMP were labeled for 1 h by incubation at 37°C in medium containing 1 μCi/ml of [*methyl*-^{3}H]thymidine (spec. act. 78.3 Ci/mmol, NEN). Cells were then collected by a brief trypsinization and centrifugation. The cell pellet was washed once with 10 ml cold phosphate-buffered saline (PBS). The final cell pellet was resuspended in 1 ml cold PBS and an equal volume of 20% (w/v) ice-cold trichloroacetic acid was added to it and left on ice for 10 min. Collection of trichloroacetic acid-precipitable material and radioactivity counting were as described [15].

3. RESULTS AND DISCUSSION

The growth rate of F9 cells was determined by [^{3}H]thymidine uptake into DNA and by parallel cell counting at various intervals after initiation of induction with RA and cAMP. As shown in fig.1, the growth rate of F9 cells after 24 and 48 h of RA and cAMP induction did not change significantly as compared to that of uninduced cells. However, following 72 h of induction with RA and cAMP, the cells showed a markedly decreased growth rate. These decreases in F9 cell growth rates were even more prominent after 96 and 120 h of induction with RA and cAMP (fig.1). Our results on F9 cell growth rate during the course of RA and cAMP induction are in excellent agreement with those of Campisi et al. [14]. The viability of F9 cells even after prolonged periods of induction with RA and cAMP (in this case 120 h) was above 90%. Furthermore, these cells had a protein synthetic activity, as determined by [^{14}C]leucine uptake into trichloroacetic acid-precipitable proteins, of 80% of that of uninduced cells.

In order to study the regulation of expression of the CAD gene during cell growth, steady-state levels of CAD mRNA were determined in F9 cells at various intervals of exponential growth and time points after the initiation of induction with RA and cAMP. Steady-state levels of CAD mRNA in uninduced exponentially growing F9 cells were not affected significantly during the experimental period (not shown). As shown in fig.2, no significant changes were observed in the steady-state levels of CAD mRNA in 24 and 48 h RA- and cAMP-induced cells as compared to levels in unin-

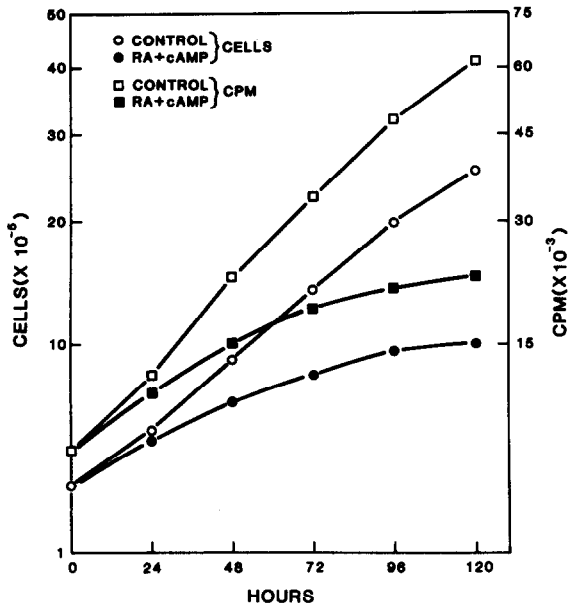

Fig.1. Growth rate of F9 teratocarcinoma cells in the presence and absence of the inducers, RA and cAMP.

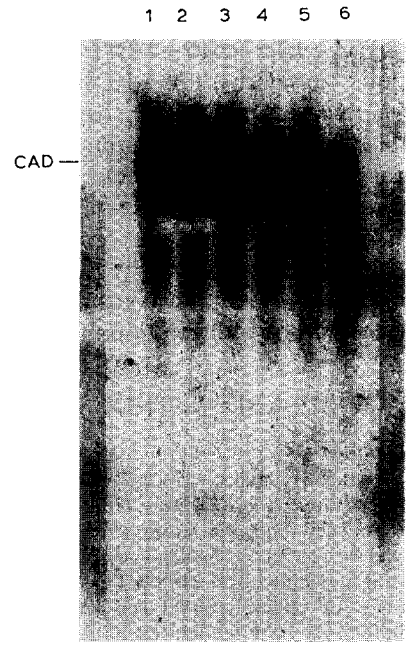

Fig.2. Northern blot analysis of CAD mRNA. Equal amounts of total cellular RNA (40 μg/lane) from exponentially growing (lane 1) and from 24, 48, 72, 96 and 120 h RA- and cAMP-induced F9 cells (lanes 2–6, respectively) were fractionated electrophoretically on agarose-formaldehyde gel, transferred to nitrocellulose filters and hybridized with nick-translated pCAD142 DNA.

duced cells. However, at 72 to 120 h of induction with RA and cAMP, the respective steady-state levels of CAD mRNA decreased by 3- and 7-fold vs levels in uninduced F9 cells (fig.2). On the other hand, when the Northern blot was reprobed with β-actin probe, no differences were found in its steady-state mRNA levels between induced and uninduced cells. In fact, a slight increase in mRNA content was observed in cells induced for 96 and 120 h (fig.3). This result indicates that decreased CAD mRNA levels in induced F9 cells are not due to a general effect of the inducers but specifically reflect the decreased cellular growth rate. The decreases in CAD mRNA steady-state levels from 72 to 120 h of RA and cAMP induction are in accordance with the decreased cellular growth rates observed at those time points of induction. This positive correlation between decreased CAD steady-state mRNA levels and decreased cellular growth rates suggests that CAD gene expression is linked to cellular proliferation. Another line of evidence in support of CAD gene expression being linked to cellular proliferation comes from experiments using BALB/c 3T3 cells. When these cells were synchronized by serum deprivation and then stimulated with serum, CAD steady-state mRNA levels increased 10-fold (Rao and Church, unpublished).

To delineate the mechanism for the down regulation of CAD steady-state mRNA levels in induced F9 cells, nuclei were isolated from 120 h RA- and cAMP-induced cells and from exponentially growing cells (48 h after seeding), followed by performing nuclear run-off assays. At these time points the densities of induced and uninduced F9 cells were about the same ($6–8 \times 10^5$ cells/ml) but steady-state CAD mRNA levels differ from each other by a factor of 7. The nuclear run-off assay determines the number of nuclear transcripts present on the chromatin for a particular gene at a given time. Nuclear transcripts initiated in vivo and elongated in vitro were hybridized to CAD and β-actin DNAs immobilized on nitrocellulose filters (fig.4). Note that the rate of CAD gene transcription between 120 h RA- and cAMP-induced and uninduced F9 cells was unchanged. The rate of β-actin gene transcription between induced and unin-

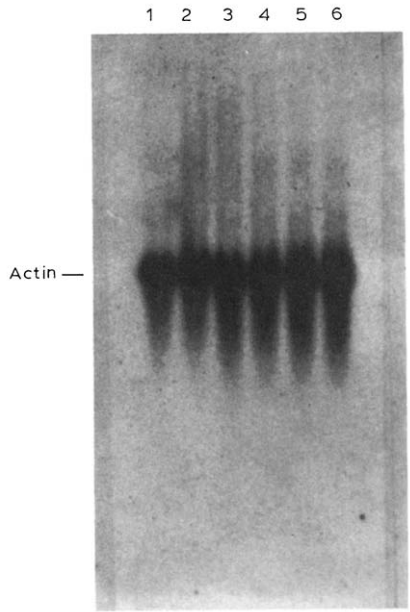

Fig.3. Northern blot analysis of β-actin mRNA. The Northern blots which were used for pCAD142 probing were reprobed with β-actin probe, pHF5, as described in section 2.

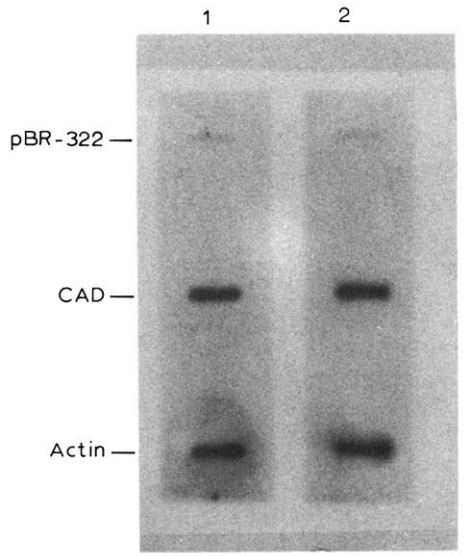

Fig.4. Transcriptional analysis of CAD and β-actin genes in exponentially growing (lane 1) and 120 h RA- and cAMP-induced (lane 2) F9 cells.

duced F9 cells also remained unchanged, which in fact agrees with its steady-state mRNA levels. It is most unlikely that the nuclear run-off signals with CAD and β-actin DNAs are artifactual, since no hybridization signals were observed with control pBR322 DNA (fig.4). At the same time, when the nuclear transcripts were isolated in the presence of α-amanitin, a potent inhibitor of RNA polymerase II activity, and hybridized to CAD and β-actin gene DNAs, no signals were found (not shown). This result further indicates that the transcripts hybridized to CAD and β-actin gene DNAs are RNA polymerase II activity dependent. The decreased steady-state CAD mRNA levels in induced F9 cells compared to the unaltered rate of its transcription between induced and uninduced cells suggests a role for posttranscriptional mechanisms such as RNA processing or RNA stability during RA- and cAMP-induced differentiation of F9 cells. However, previously we [15] and Lio et al. [23] reported a down regulation of CAD gene in terminally differentiating HL-60 cells and in serum-starved Syrian hamster cells, respectively. We [15] and Liao et al. [23] have further reported that the down regulation of the CAD gene in those systems is due to a parallel decrease in its transcription rate. Although the present and previous studies [15,23] clearly demonstrate a down regulation of the CAD gene during cellular growth arrest, it appears that the mechanisms responsible for its down regulation vary among different cell types. This type of regulation may not be unique to the CAD gene alone, since such types of regulation have also been reported for other genes such as thymidine kinase [24–26], dihydrofolate reductase [27–29], and *c-myc* [21,30]. For example, in the case of *c-myc*, transcriptional mechanisms were reported for its down regulation in differentiating HL-60 cells [21] whereas posttranscriptional mechanisms were described for its down regulation in differentiating F9 cells [30].

ATCase activity was used as a general marker of the three enzymes associated with CAD protein. Its activity in 120 h RA- and cAMP-induced F9 cells decreased by 50% vs uninduced cells (table 1). This is a relatively small decrease in ATCase activity as compared to the 7-fold decrease in its message content in induced cells, which could be due to a long half-life of CAD protein in F9 cells. A half-life of 75 h for the CAD protein was reported in Syrian hamster cells [31]. Our results on CAD mRNA levels and ATCase activity in F9 cells during induc-

Table 1

ATCase activity in F9 teratocarcinoma cells during RA- and cAMP-induced differentiation

Induction time (h)	ATCase activity
0	11.62
24	12.46
48	11.37
72	10.86
96	8.12
120	5.68

ATCase activity is expressed as nmol *N*-carbamoyl L-aspartate formed/mg protein per min

tion with RA and cAMP are in agreement with those of Liao et al. [23] in serum-starved Syrian hamster cells.

The down regulation of the CAD gene in differentiating F9 cells (present findings) and differentiating HL-60 cells [15] together with the previously observed positive correlation between the activities of the enzymes associated with the CAD gene and the degree of cellular growth [2–7] strongly support our hypothesis that the de novo pyrimidine biosynthetic activity is linked to cellular proliferation. Since CPSase is the first and rate-limiting enzyme of de novo pyrimidine biosynthesis in higher eukaryotes and one of the three enzymes encoded by the CAD gene, it is clear from the above findings that de novo pyrimidine biosynthesis during cellular growth is regulated by modulating the synthesis of its rate-limiting enzyme, CPSase.

REFERENCES

[1] Shoaf, W.T. and Jones, M.E. (1973) Biochemistry 12, 4039–4051.
[2] Galofre, A. and Kretchmer, N. (1970) Pediatr. Res. 4, 55–62.
[3] Kim, S. and Cohen, P.P. (1965) Arch. Biochem. 109, 421–428.
[4] Nordmann, Y., Hurwitz, R. and Kretchmer, N. (1964) Nature 201, 616–617.
[5] Shambaugh, G.E., Mrozak, S.C., Metzger, B.E. and Freinkel, N. (1974) Dev. Biol. 37, 171–185.
[6] Jones, M.E. (1972) Curr. Top. Cell. Regul. 6, 227–285.
[7] Aoki, T., Morris, H.P. and Weber, G. (1982) J. Biol. Chem. 257, 432–438.
[8] Chen, J.J. and Jones, M.E. (1979) J. Biol. Chem. 254, 4908–4914.
[9] Hoogenraad, N.J. and Lee, D.C. (1974) J. Biol. Chem. 249, 2763–2768.
[10] Monks, A., Anderson, L.W., Strong, J. and Cysyk, R.L. (1983) J. Biol. Chem. 258, 13564–13569.
[11] Shigesada, K., Stark, G.R., Maley, J.A., Niswander, L.A. and Davidson, J.N. (1985) Mol. Cell. Biol. 5, 1735–1742.
[12] Strickland, S. and Mahdavi, V. (1978) Cell 15, 393–403.
[13] Strickland, S., Smith, K.K. and Marotti, K.R. (1980) Cell 21, 347–355.
[14] Campisi, J., Gray, H.E., Pardee, A.B., Dean, M. and Sonenshein, G.E. (1984) Cell 36, 241–247.
[15] Rao, G.N., Buford, E.S. and Davidson, J.N. (1987) Mol. Cell. Biol. 7, 1961–1966.
[16] Chirgwin, J.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[17] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[18] Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. and Kedes, L. (1983) Mol. Cell. Biol. 3, 787–795.
[19] Meinkoth, J. and Wahl, G.M. (1984) Anal. Biochem. 138, 267–284.
[20] Groudine, M., Peretz, M. and Weintraub, H. (1981) Mol. Cell. Biol. 1, 281–288.
[21] Linial, M., Gunderson, N. and Goudine, M. (1985) Science 230, 1126–1132.
[22] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[23] Liao, W.S.L., Heller, R., Green, P. and Stark, G.R. (1986) J. Biol. Chem. 261, 15577–15581.
[24] Groudine, M. and Casimir, C. (1984) Nucleic Acids Res. 12, 1427–1446.
[25] Gross, M.K., Kainz, M.S. and Merrill, G.F. (1987) Dev. Biol. 122, 439–451.
[26] Stewart, C.J., Ito, M. and Conrad, S.E. (1987) Mol. Cell. Biol. 7, 1156–1163.
[27] Leys, E.J. and Kellems, R.E. (1981) Mol. Cell. Biol. 1, 961–971.
[28] Kaufman, R.J. and Sharp, P.A. (1983) Mol. Cell. Biol. 3, 1598–1608.
[29] Santiago, C., Collins, M. and Johnson, L.F. (1984) J. Cell. Physiol. 118, 79–86.
[30] Dony, C., Kessel, M. and Gruss, P. (1985) Nature 317, 636–639.
[31] Padgett, R.A., Wahl, G.M., Coleman, P.F. and Stark, G.R. (1979) J. Biol. Chem. 254, 974–980.