INDUCTION OF TYROSINE AMINOTRANSFERASE BY
DIBUTYRYL CYCLIC-AMP IN SYNCHRONIZED HEPATOMA CELLS

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ABSTRACT

In Hepatoma Tissue Culture (HTC) cells induction of tyrosine
aminotransferase (TAT) by dibutyryl cAMP (DBcAMP) is regulated at
some posttranscriptional step. In synchronized HTC cells TAT can
be induced by DBcAMP in late G_1 and in the S phase of the cell
cycle only.

INTRODUCTION

In rat Hepatoma Tissue Culture (HTC) cells, induction of tyro-
sine aminotransferase (TAT) by serum (Gelehrter and Tomkins, 1969),
insulin (Gelehrter and Tomkins, 1970) or corticosteroid hormones
(Thompson et al., 1966; Tomkins et al., 1969) has been documented
extensively. Although the inducibility of TAT by $N^6,O^{2'}$ dibutyryl
cyclic AMP (DBcAMP) has been the subject of some doubt (Butcher et
al., 1971; Granner, 1976), various authors observed a small, but
consistent increase in TAT specific activity after addition of this
compound (Stellwagen, 1972; van Rijn et al., 1974).
We considered it of interest to complete our knowledge on the
effect of the various inducers in the different phases of the cell
cycle. Martin et al. (1969b) and Sellers and Granner (1974) repor-
ted that induction of TAT by dexamethasone is only possible during
passage of cells through late G_1 and S phases. On the other hand
Emanuel and Gelehrter (1975) observed that induction of TAT by in-
sulin occurs immediately upon entry of mitotic cells into G_1, and
is maintained during passage of the cells through G_1 and S phases.
In this paper our earlier results on the induction of TAT by DBcAMP
(van Rijn et al., 1974) have been extended with data showing that
induction is only possible in cells in the late G_1 and in the S
phase of the cell cycle. These results once more indicate an inter-
relationship between the mechanism by which corticosteroids and
cyclic AMP regulate TAT synthesis.

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MATERIALS AND METHODS

HTC cells (Thompson et al., 1966) were grown as monolayer cultures (doubling time 22-24 hr) in plastic Falcon flasks. The medium consisted of Eagle's basal medium enriched 4-fold with vitamins and amino acids, and buffered at pH 7.4. Fetal calf serum and calf serum were added to a final concentration of 5 and 10% respectively. For synchronization the procedure of Martin et al. (1969b) was slightly modified: log phase cultures of HTC cells were shaken vigorously in order to remove loosely attached cells, and fresh medium containing colcemid (final concentration 2 x 10^{-7} M) was added. After 8 hr the medium was removed carefully and the mitotic cells were collected by gently swirling the plates with fresh medium. The mitotic index of these cells, determined after fixation and Feulgen staining (van Wijk et al., 1977) was always higher than 90%. Mitotic cells were seeded in new flasks at a density comparable to the growth conditions, and were found to be attached approximately 2 hr later.

G_2 cells were analyzed as described by Martin et al. (1969b). According to this procedure induction of TAT by DBcAMP in G_2 cells was measured by addition of both colcemid and DBcAMP to cultures, and collecting mitotic cells at 2 hr intervals. For this procedure Prescott (1976) suggested the term retroactive synchronization.

The percentage of cells in S-phase was determined by autoradiography, after incorporation of $|^3H|$thymidine (44 mCi per μmole, 0.2 μCi per ml of culture medium) during 30 min, and fixation (van Wijk et al., 1977). In the retroactive synchronization procedure the cultures were pulsed with $|^3H|$thymidine, then washed twice with growth medium, and incubated in medium containing colcemid. Autoradiography was performed on the mitotic cells.

Assays

Mitotic cells were collected by centrifugation at 500 g during 5 min in the cold, and washed twice with icecold 0.9% NaCl. Interphase cells and unsynchronized populations were harvested by washing the monolayer twice with icecold 0.9% NaCl and then scraping it with a rubber policeman. Lysis of the cells was performed in the presence of 0.1% nonidet (Shell). Extracts were centrifuged (5 min, 5000 g), and TAT activity was determined according to Diamondstone (1966). Protein was determined according to Lowry et al. (1951). The rate of protein synthesis was measured by pulse-labeling with $|^3H|$leucine (105 mCi per μmole, 1 μCi per ml culture medium) during 30 min. Extracts were prepared as described above, and $|^3H|$leucine incorporated into protein was determined as described by Mans and Novelli (1961). In the retroactive synchronization procedure cell cultures were labeled with $|^3H|$leucine for 30 min, then washed twice with fresh growth medium, and subsequently incubated in medium containing colcemid. Ultimately, the incorporated $|^3H|$leucine was determined in the collected mitotic cells.
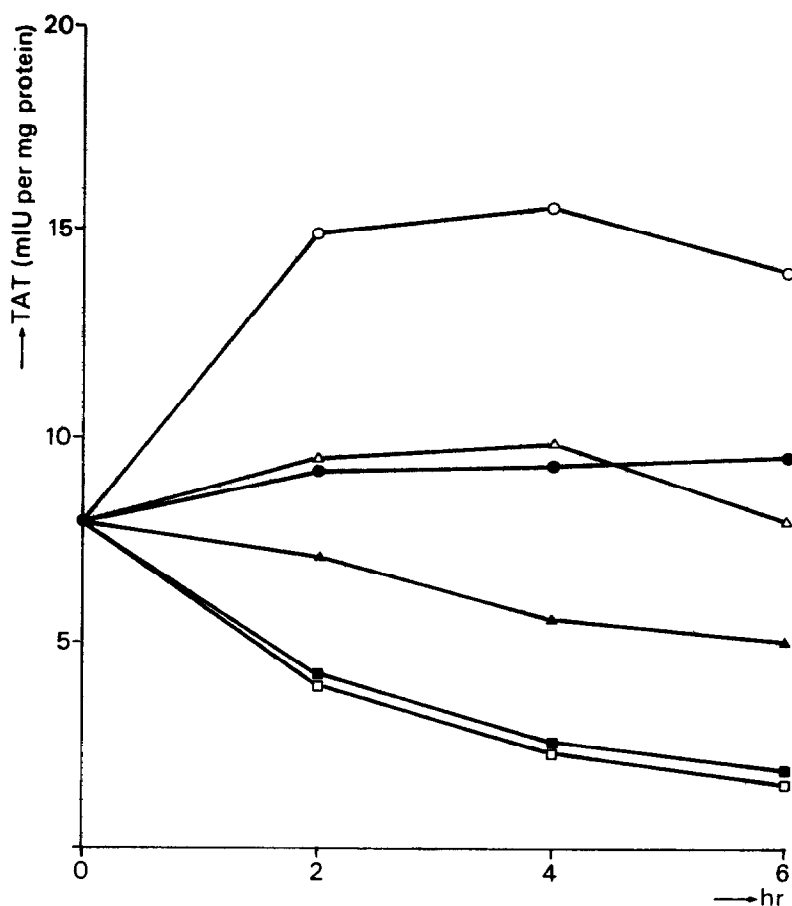
Figure 1. *Induction of TAT by DBcAMP in a random culture of HTC cells.* DBcAMP (final concentration 1 mM) and cycloheximide (final concentration 0.3 mM) were added to the medium at zero time, and actinomycin D (final concentration 0.3 µg per ml) was added one hr before zero time. (●—●), no additions; (o—o), in the presence of DBcAMP; (▲—▲), in the presence of actinomycin D; (△—△), in the presence of DBcAMP and actinomycin D; (■—■), in the presence of cycloheximide; (□—□), in the presence of DBcAMP and cycloheximide.

Chemicals

Radioactive chemicals were from the Radiochemical Centre, Amersham, England. All tissue culture components were from Gibco Grand Island Biological Co. (Grand Island, N.Y.). $N^6, O^{2'}$ dibutyryl cyclic 3',5'AMP was obtained from Boehringer Mannheim.

RESULTS

Induction of TAT by DBcAMP in a logarithmically growing culture

      The occurrence of induction of TAT by DBcAMP (1 mM) in HTC
cells was verified using a randomly growing culture of HTC cells
(Fig. 1). At this concentration DBcAMP has no effect on over-all
protein synthesis (Stellwagen, 1972). Cycloheximide at a concen-
tration of 0.3 mM inhibits protein synthesis by 90% or more (Peter-
kofsky and Tomkins, 1968). The results with this compound indicate
that induction depends on continuous protein synthesis, and in ac-
cordance with a high turnover of TAT (Auricchio et al., 1969) the
basal level of this enzyme is decreased. Also in the presence of
actinomycin D (0.3 µg per ml culture medium results in an inhibi-
tion of RNA synthesis of 90% or more (Peterkofsky and Tomkins, 1968))
we observed a decrease of TAT, which is in agreement with a high
turnover of the TAT mRNA (Steinberg et al., 1975), but induction of
TAT by DBcAMP is still possible in the presence of actinomycin D.

Characterization of the cell cycle of synchronized HTC cells

      A characterization of the cell cycle of the synchronized cells
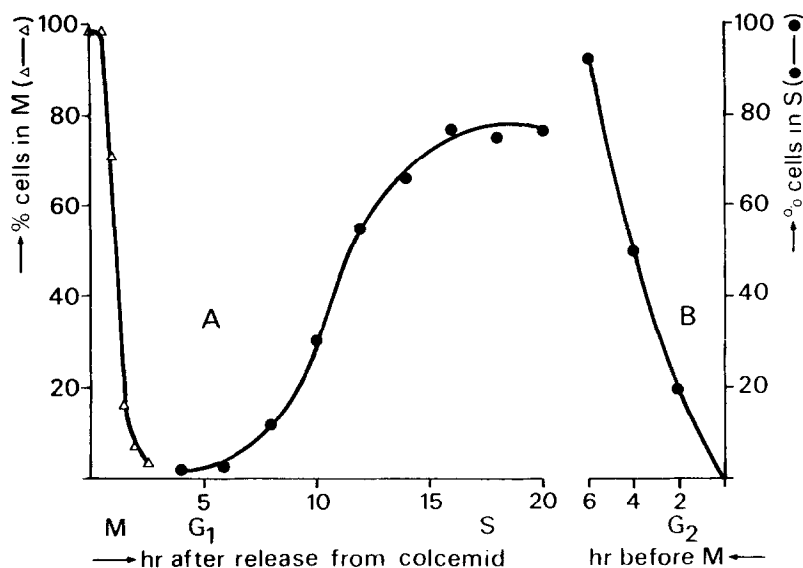is presented in Fig. 2.

Fig. 2. *The cell cycle of a synchronous population of HTC cells.*
        Fig. 2A. Cells were synchronized by mitotic shake-off as
        described in the section on Methods. Fig. 2B. Cells were
        analyzed by retroactive synchronization. S phase is ex-
        pressed as the fraction of cells harvested in mitosis,
        which are positive in autoradiography.

Mitotic HTC cells, free of colcemid, rapidly complete mitosis and
enter G_1 (Fig. 2A). The degree of synchrony is very high in mitosis
and early G_1, but afterwards it decreases due to the variability in
G_1 phase durations of individual cells (van Wijk et al., 1977).
With the aid of microcinematography completion of the cell cycle
was followed for 76 cells. It appeared that 72 cells completed mi-
tosis, and the mean generation time was found to be 24 hr, which is
in good agreement with the generation time of 22-24 hr observed in
the random culture.

Since the mean duration of the G_2 phase is only 4 hr (van Wijk
et al., 1977), the percentage of cells in this phase of the cell
cycle at any time is too low to obtain meaningful results. There-
fore cells were analyzed by retroactive synchronization (Fig. 2B).

Induction of TAT by DBcAMP in synchronized cells

Since the mitotic phase is relatively short as compared to the
duration of the induction process, induction of TAT by DBcAMP was
determined in mitotic cells in the continuous presence of colcemid.
During a 6 hr experiment DBcAMP induction was completely absent in
these cells (colcemid itself did not influence induction of TAT by
DBcAMP in a logarithmically growing culture) (results not shown).

Induction of TAT in G_1 and S phase cells was performed by the
addition of DBcAMP to synchronized HTC cultures at various times
after release from colcemid. From Fig. 3A it is clear that induc-
tion by DBcAMP does not occur during mitosis and early G_1, but is
optimal in late G_1 and S. From the analysis of G_2 cells by retroac-
tive synchronization (Fig. 3B) induction of TAT by DBcAMP appears
to be absent in this phase of the cell cycle.

Since induction of TAT by DBcAMP is dependent on protein syn-
thesis, the rate of |^{3}H|leucine incorporation into protein was mea-
sured throughout the cell cycle (Fig. 2). In accordance with the
results of Martin et al. (1969b) it was found that protein synthesis
is quite constant during interphase, including early G_1 and G_2, but
is substantially lowered in mitotic cells.

DISCUSSION

Induction of TAT by DBcAMP in HTC cells is presumed to be a cy-
clic AMP regulated process. In this respect data on the metabolism
of DBcAMP in HTC and other hepatoma cell lines (Bevers et al., 1976)
agree with the finding that TAT activity is increased in response to
several other cyclic nucleotide derivatives, i.e. 8-benzylthio cAMP,
8-methylthio cAMP and 8-ethylthio cAMP, while butyrate showed no spe-
cific effect (Stellwagen et al., 1977).

The induction of TAT by DBcAMP in HTC cells is dependent on con-
tinuous protein synthesis, and in accordance with a high rate of turn-
over of TAT, the basal level of this enzyme declines in the presence
of cycloheximide. In agreement with a high rate of turnover of the
TAT mRNA the basal level of TAT decreases also in the presence of
actinomycin D, but DBcAMP can still induce TAT. Complicating effects
such as superinduction are generally observed at higher concentrations
of actinomycin D (Thompson et al., 1970). Based on our present results

it appears that DBcAMP acts at some posttranscriptional step. A similar conclusion has been drawn earlier for the induction of TAT in HTC cells by insulin (Gelehrter and Tomkins, 1970), or by a serum factor (Gelehrter and Tomkins, 1969). Comparable observations have been reported for induction of TAT in H35 hepatoma cells by DBcAMP and by insulin (Wicks et al., 1973). RNA synthesis is required, however, for the induction of TAT by dexamethasone in both H35 (Wicks et al.,' 1973) and HTC (Tomkins et al., 1969).
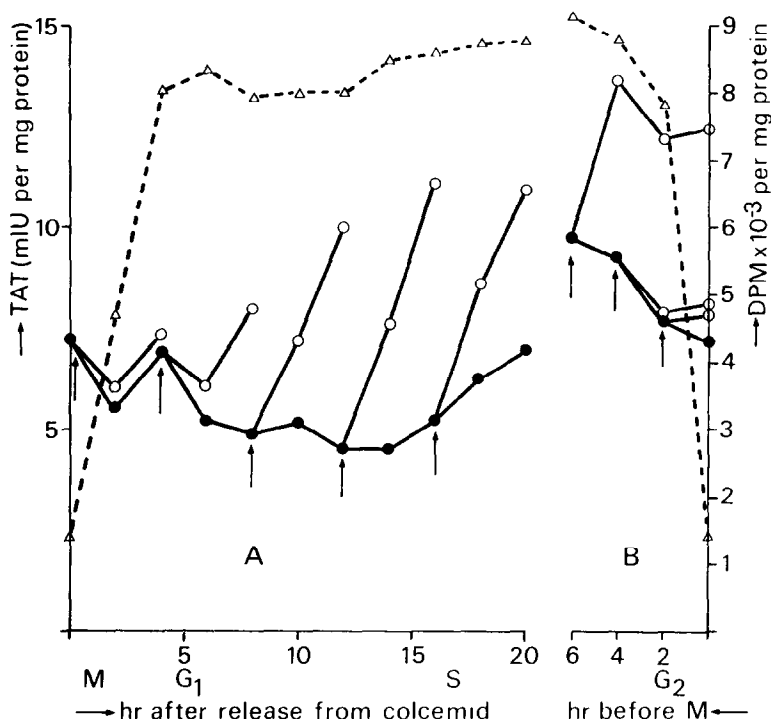

Figure 3. *Induction of TAT by DBcAMP, and over-all protein synthesis, in a synchronous population of HTC cells.* At the times indicated by the arrows DBcAMP was added to the medium to a final concentration of 1 mM. (●—●), no additions; (o—o), in the presence of DBcAMP. Over-all protein synthesis was determined as the incorporation of |^{3}H|leucine (△--△) as described in the section on methods. Cells were synchronized by mitotic shake-off (Fig. 3A), or by retroactive synchronization (Fig. 3B). Since in the latter procedure only cumulative induction can be measured, the intermediate values for induction were calculated by subtracting from the 4 and 6 hr time points the induced TAT values determined in the previous 2 hr period(s).

The experiments described in this paper prove that TAT can be induced by DBcAMP in late G_1 and in the S phase of the cell cycle only.

Absence of induction in M is probably due to a substantially lowered
rate of protein synthesis, a phenomenon that has been observed be-
fore (Martin et al., 1969b). The absence of induction by DBcAMP in
early G_1 and in G_2 phase cells is remarkably different from the sen-
sitivity of these cells towards insulin (Emanuel and Gelehrter,
1975): apparently at least two different mechanisms of posttrans-
criptional stimulation of TAT synthesis do occur in the cell. On
the other hand the absence of induction by DBcAMP is quite compa-
rable to the absence of induction by dexamethasone in early G_1 and
G_2 (Martin et al., 1969b; Sellers and Granner, 1974). Apparently,
both transcription of the TAT gene(s) (Martin et al., 1969b; Martin
et al., 1969a; Martin and Tomkins, 1970; Sellers and Granner, 1974)
and posttranscriptional stimulation by DBcAMP are limited to the
late G_1 and S phases in the cell cycle of HTC cells.
      These results stress the interrelationship between DBcAMP and
corticosteroid hormone induction of TAT. Such an interrelationship
has also been suggested from experiments showing a "permissive ef-
fect" of corticosteroids on the induction of TAT by DBcAMP in rat
liver and liver derived systems, including HTC (Granner, 1976). How-
ever, our results make it likely that DBcAMP induction of TAT is not
absolutely dependent on the presence of corticosteroids. Results on
the mechanism of DBcAMP induction are contradictory: in rat liver the
amount of mRNA for TAT is increased in the presence of DBcAMP (Ernest
and Feigelson, 1978; Noguchi et al., 1978), but in H35 hepatoma cells
a decrease in the transit time for TAT fully accounts for the increase
in TAT activity (Roper and Wicks, 1978). For hepatoma cell lines it
can be assumed that the DBcAMP inducing system, stimulating transla-
tion of the TAT mRNA, is only active during late G_1 and S phase. Ab-
sence of induction by dexamethasone in G_2, M and early G_1 can be ex-
plained by an absolute dependence of translation of the TAT mRNA on
the DBcAMP inducing system, which in fact is observed in some liver
systems (Ernest et al., 1978). At present it is under investigation
whether the DBcAMP inducing system directly or indirectly could be
responsible for the observed increase in TAT mRNA in the presence of
DBcAMP in rat liver.

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