Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1254-1258

IN VITRO TRANSLATION OF EPOXIDE HYDRATASE MESSENGER RNA[1]

Frank J. Gonzalez and Charles B. Kasper

McArdle Laboratory for Cancer Research
University of Wisconsin
Madison, Wisconsin 53706

Received March 15,1980

SUMMARY. Rat liver epoxide hydratase mRNA, found exclusively associated with polysomes tightly bound to the endoplasmic reticulum, was translated using a rabbit reticulocyte protein synthesizing system. Epoxide hydratase synthesized in vitro was found to be identical to the native enzyme in terms of molecular weight and partial proteolytic peptide patterns, indicating the absence of a cleavable signal sequence. Translatable levels of epoxide hydratase mRNA increased 3-fold at 4 hours after phenobarbital administration. This increase was inhibited by cordycepin, an RNA synthesis chain terminator. These results demonstrate that epoxide hydratase is induced by phenobarbital through transcriptional and/or post-transcriptional nuclear events.

Recently it was reported that phenobarbital induction of a species of cytochrome P-450 may result from an increase in its mRNA; however, the maximum level of translatable mRNA did not occur until 12 h (1) to 16 h (2) after a single dose of the drug. The long interval between administration of phenobarbital and the peak in mRNA level suggests that the drug may increase cytochrome P-450 mRNA by causing a decrease in its rate of degradation. In contrast, others (3) have reported that translatable levels of cytochrome P-450b mRNA increases as early as 6 hours after phenobarbital administration; however, the total protein translated was also increased by a similar magnitude, suggesting that cytochrome P-450b mRNA was not specifically induced. In order to explore the mechanism by which phenobarbital induces certain enzymes involved in drug metabolism, translatable levels of mRNA coding for epoxide hydratase, a phenobarbital inducible enzyme (4), were examined after phenobarbital administration. In addition, the site of synthesis of epoxide

[1] This research was supported by grants CA-23076 and CA-17300 from the National Cancer Institute, Department of Health, Education and Welfare.

[2] Abbreviations used: SDS, sodium dodecyl sulfate.

0006-291X/80/081254-05$01.00/0

hydratase within the cell is examined, and the enzymes synthesized in vitro and in vivo are compared using a partial protelolytic mapping procedure.

MATERIALS AND METHODS

Preparation of Epoxide Hydratase and Anti-epoxide Hydratase Gamma Globulin. Epoxide hydratase was purified according to the methods of Knowles and Burchell (5). The purified enzyme was concentrated and separated from detergent using hydroxylapatite (B10-RAD) as described by Lu et al. (6). The molecular weight of the purified enzyme was determined by using the technique of Weber and Osborne (7). Antisera to epoxide hydratase was raised in adult male New Zealand albino rabbits and gamma-globulin prepared as described (8).

Animals. Male Sprague-Dawley rats weighing 180-200 gms were injected in a caudal tail vein with a 10% solution of sodium phenobarbital in 0.15 M NaCl and a dose of 100 mg/kg. Cordycepin (3 mg/100 gm) was injected intraperitoneally.

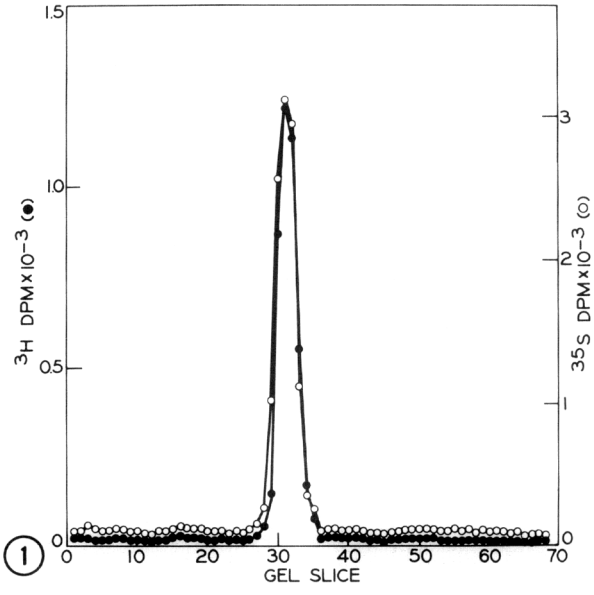
In Vitro Translation and Quantitation of Translatable Epoxide Hydratase mRNA. In vitro translation was carried out according to the procedure of Pelham and Jackson (9) using ^{35}S-methionine (Amersham) as the label. The reaction was carried out in a volume of 68 ul, at 30°C for 45 min and terminated by addition of a 10% triton X-100 and 10% deoxycholate solution to a 1% final concentration. The reaction mixtures were then sedimented at 90,000 xg for 2 h and the radioactivity in trichloroacetic acid precipitable polypeptides was determined from a 2 ul aliquot of the supernatant. Newly synthesized epoxide hydratase was immunoprecipitated using 10 ug of carrier and the washed immunoprecipitate electrophoresed on a 10% SDS^2-polyacrylamide gel (10). The band corresponding to epoxide hydratase was excised, dissolved in protosol (New England Nuclear Corp.) and counted using RIA liquid scintillation fluid (Research Products International Corp.). Partial proteolytic mapping of epoxide hydratase was carried out as described (11), using 10 ug of Staph. aurease protease (Miles Biochemicals) and 0.8 mg of chymotrypsin.

Preparation of Polysomes and Isolation of RNA. Membrane-bound and free polysomes were isolated from post mitochondrial supernatant as described (12), except that the membrane was first washed with 0.5 M KCl before releasing the polysomes with 1% Triton X-100 and 1% deoxycholate in the presence of 0.5 mg/ml heparin. The released polysomes were sedimented through a 2 M sucrose cushion as described (12). RNA was then extracted with phenol-chloroform (13) and after precipitation with 2 vol of ethanol and washing with 3 M sodium acetate (pH 6.0), the RNA was dialyzed exhaustively against doubly distilled water and lyophilized to dryness. RNA, which was used at a concentration of 750 ug/ml, stimulated translation to 15-20 fold above background.

RESULTS AND DISCUSSION

In Vitro Translation of Epoxide Hydratase. Only mRNA isolated from membrane-bound polysomes coded for epoxide hydratase in the reticulocyte cell free translation system. Epoxide hydratase was not detected in free polysomal RNA translation products. The enzyme synthesized in vitro comi-

grated on an SDS-polyacrylamide gel with the *in vivo* synthesized enzyme immunoprecipitated from [^{3}H]leucine labeled endoplasmic reticulum with an estimated molecular weight of 53,000 (Fig. 1). The *in vitro* and *in vivo* synthesized enzymes were further shown to be identical by mapping the partial proteolytic fragments produced by chymotrypsin and *Staph. aureas* protease (Fig. 2). Bands A, B, C and D of the Coomassie blue stained *Staph. aureas* protease map (lane 1), which represent the enzyme synthesized *in vivo*, comigrated with ^{35}S-labeled peptide fragments produced from the enzyme synthesized *in vitro* (lane 2). In the autoradiographic pattern (lane 2),



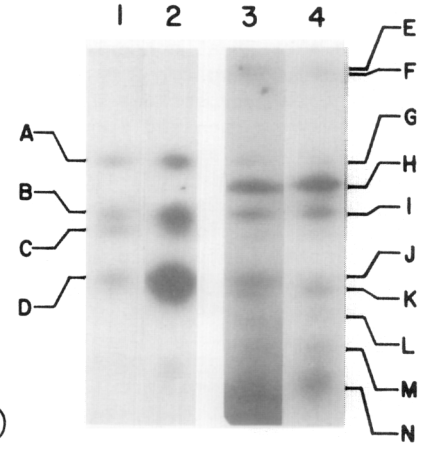


Figure 1. SDS-polyacrylamide gel electrophoresis of *in vitro* and *in vivo* synthesized epoxide hydratase. Immunoprecipitates of epoxide hydratase synthesized *in vivo* (-●-●-) and *in vitro* (-○-○-) were combined and co-electrophoresed on an SDS-polyacrylamide gel. The gel was sliced into 2 mm sections, processed and counted as described in "Materials and Methods". Approximately 1.4 x 10^{7} DPM of total released protein coded for by 4-h phenobarbital-induced RNA was used for immunoprecipitation of the enzyme synthesized *in vitro*.

Figure 2. Electrophoretic comparison of proteolytic fragments derived from *in vitro* and *in vivo* synthesized epoxide hydratase. An immunoprecipitate of *in vitro* synthesized epoxide hydratase and the purified enzyme were digested with *Staph. aurease* protease (lanes 1 and 2) or chymotrypsin (lanes 3 and 4) in an SDS-polyacrylamide gel, and the partial proteolytic fragments separated on a slab gel as described (11). Lanes 1 and 3 contain a Coomassie blue stain of proteolytic fragments produced from purified epoxide hydratase and lanes 2 and 4 contain an autoradiograph of the proteolytic fragments produced from the *in vitro* synthesized enzyme.

fragments B and C are not well resolved; however, this appears to be due to the disproportionately higher amount of label in peptide B. In the case of the chymotrypic map, all Coomassie blue stained fragments (lane 3) also comigrated with ^{35}S-labeled fragments (lane 4), with peptides H and I representing major cleavage products. These results strongly suggest that epoxide hydratase is not synthesized in a form detectably larger than the native enzyme. Since the average length of known "signal" peptides is from 15-20 amino acids (14) the presence of a cleavable signal sequence would readily be detected by SDS-polyacrylamide gel electrophoresis and partial peptide mapping. Thus, even though epoxide hydratase is synthesized on membrane-bound polysomes, it may not be inserted into the endoplasmic reticulum via a cleavable "signal sequence" (15). A similar conclusion was reached in the case of cytochrome P-450, in which no difference was noted between the *in vitro* and *in vivo* synthesized enzyme (16).

Phenobarbital Induction of Epoxide Hydratase mRNA. Translatable levels of epoxide hydratase mRNA specifically increased 3-fold at 4 hours after administration of phenobarbital (Table I). This increase was inhibited

TABLE I

Phenobarbital Induction of Epoxide Hydratase mRNA[1]

Membrane-Bound Polysomal RNA	Percent of Total Polysome Released Protein Represented by Epoxide Hydratase[2]
Control	.023 ± .003
Phenobarbital Induced	.076 ± .004
Phenobarbital plus cordycepin	.031 ± .007

[1] *In vitro* translation was carried out using the various RNA preparations. Epoxide hydratase was immunoprecipitated from approximately 2.5 x 10^6 DPM of total polysome released protein and the washed immunoprecipitate electrophoresed on an SDS-polyacrylamide gel. The Coomassie-blue stained band corresponding to epoxide hydratase was excised and the radioactivity in the band determined. Four regions of the gel above and below the band were counted for background radioactivity determinations.

[2] Mean ± standard deviations of three separate experiments.

when the RNA synthesis chain terminator cordycepin was administered 20 min before the drug. The rapid increase in translatable epoxide hydratase mRNA after administration of phenobarbital is not consistent with the hypothesis that the drug expands mRNA pools solely through mRNA stabilization (17,18). Interestingly, hydrocortisone also causes a 3-fold increase in translatable levels of tyrosine aminotransferase and tryptophan oxygenase mRNAs as early as 4 hours after administration (19), suggesting that glucocorticoids and phenobarbital may have similar modes of action. Furthermore, since cordycepin has been shown to inhibit accumulation of newly synthesized mRNA (20) its blocking action on the induction process indicates that phenobarbital may increase epoxide hydratase mRNA levels via transcriptional and/or post-transcriptional nuclear events.

REFERENCES

1. Dubois, R.N., and Waterman, M.R. (1979) Biochem. Biophys. Res. Commun. 90, 150-157.
2. Kumar, A., and Padmamabon, G. (1980) J. Biol. Chem. 255, 522-525.
3. Colbert, R.A., Bresnick, E., Levin, W., Ryan, E.D., and Thomas, R.E. (1979) Biochem. Biophys. Res. Commun. 91, 886-891.
4. Bresnick, E., Mukhtor, H., Stoming, T.A., Dansette, P.M., and Jerina, D.M. (1977) Biochem. Pharmacol. 26, 891-892.
5. Knowles, R.G., and Burchell, B. (1977) Biochem. J. 163, 381-383.
6. Lu, A.Y., Ryan, P., Jerina, D.M., Daly, J.W., and Levin, w. (1975) J. Biol. Chem. 250, 8283-8388.
7. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4405-4412.
8. Zimmerman, J.J., and Kasper, C.B. (1978) Arch. Biochem. Biophys. 190, 726-735.
9. Pelham, H.R.B., and Jackson, J. (1976) Eur. J. Biochem. 67, 247-256.
10. Laemmli, V.K. (1970) Nature (London) 277, 680-685.
11. Lam, K., and Kasper, C.B. (1979) J. Biol. Chem. 254, 11713-11720.
12. Ikehara, Y., and Pitot, H.C. (1973) J. Cell Biol. 59, 28-44.
13. Moore, R.E., Cardelli, J.A., and Pitot, H.C. (1977) Biochemistry 16, 3037-3044.
14. Leader, D.P. (1979) Trends in the Biochemical Sciences 4, 205-208.
15. Blobel, G., and Dobberstein, B. (1975) J. Cell. Biol. 67, 852-862.
16. Bar-Num, S., Kreibich, G., Adesnik, M., Alterman, L., Negishi, M., and Sabatini, D.D. (1980) Proc. Natl. Acad. Sci. USA 77, 965-969.
17. Cohen, A.M., and Ruddon, R.W. (1971) Molec. Pharmacol. 7, 484-489.
18. Venkateson, N., and Steele, W.J. (1975) Chem.-Biol. Interact. 11, 41-54.
19. Roewekamp, W.G., Hofer, E., and Seheris, C.E. (1976) J. Biochem. 70, 259-268.
20. Beach, L.R., and Ross, J. (1978) J. Biol. Chem. 253, 2628-2632.