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ALTERED FOLATE-BINDING PROTEIN mRNA STABILITY IN KB CELLS GROWN IN FOLATE-DEFICIENT MEDIUM

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Abstract—Folate-binding protein (FBP), a high-affinity folate receptor, is responsible for cellular accumulation of folate and folate analogs such as methotrexate in human KB (nasopharyngeal carcinoma) cells. Both FBP and FBP mRNA increase 3- to 5-fold when KB cells are grown in folate-deficient (less than 10 nM folate) medium (KB-FD), compared with growth in standard folate-replete medium containing at least 2 μM folate (KB-FR). The possible mechanisms of enhanced FBP gene expression in KB-FD were examined in this study. Southern blot analysis revealed no significant change in the FBP gene organization or copy number in the KB-FD DNA. While hypomethylation of the FBP gene was observed in KB-FD DNA, relative to KB-FR DNA, exposure of KB-FR to the DNA methylation inhibitors did not result in elevated FBP mRNA levels. The transcriptional rate of the FBP gene was the same in KB-FR and KB-FD. RNA half-life studies indicated that the half-life of FBP mRNA in KB-FD was increased approximately 2.5-fold, compared with KB-FR. Thus, the increase in the steady-state levels of FBP mRNA in KB-FD can be attributed partly to increased FBP mRNA stability.

Folate-binding protein (FBP‡), a folate receptor anchored to the cell membrane via a glycosyl-phosphatidylinositol linkage [1, 2], is responsible for the cellular transport of folate and folate analogs such as methotrexate in human KB (nasopharyngeal carcinoma) cells [3, 4]. Recently, FBP has been shown to be a specific tumor antigen for human ovarian cancer [5, 6]. cDNAs for FBP mRNAs have been prepared from KB cells [7, 8], human colon carcinoma cells [1], human ovarian carcinoma cells [5, 6], human placenta [9], and murine leukemia cells [10], and expression of human FBP mRNA has been detected in choroid plexus, lung, thyroid, and kidney [11]. All the cDNAs from human cancer cells have the same coding region sequence, but their 5′-untranslated regions vary and may have functional significance [6]. There are two FBPs expressed in human placenta; one of these appears to be identical to KB FBP (putative adult form) while the other (putative fetal form) shares an approximately 70% identity with KB FBP [9, 12]. Recent data have indicated that there is an FBP multi-gene family localized to chromosome 11q13, including an adult gene, a fetal gene and one or more pseudogenes [12, 13].

Folates are involved in essential one-carbon transfer reactions such as biosyntheses of purines and thymidylate and the regeneration of *S*-adenosylmethionine (SAM) for DNA methylation [14]. An increase in the demand for folate in malignant disease has been found to lead to its more rapid metabolism in cancer patients [15]. Recently, it has been shown that several malignant cell lines including KB express markedly more FBP than normal cell lines [11], and transfection of the FBP cDNA from human malignant cell lines allows FBP negative cells to grow in low folate media [16, 17]. Therefore, overexpression of FBP in cancer cells may confer a metabolic advantage for the tumor in capturing folate from serum and may facilitate rapid cell growth and division.

In standard cell-culture medium, there is generally at least 2 μM folate [3, 18], which is 40- to 400-fold higher than physiological levels of folate in human serum (5–50 nM) [19]. It has been shown that FBP levels in KB cells gradually increase several-fold during 2–3 months growth in folate-deficient (less than 10 nM folate) medium (KB-FD), compared with growth in standard folate-replete medium (KB-FR) [3, 18, 20]. The increase in FBP levels in the folate-deficient milieu is reflected in increased levels of FBP mRNA [7, 21], but the mechanism responsible for the alteration in human FBP gene expression remains unknown. In this study, several possible mechanisms for the increased FBP mRNA levels in KB-FD were investigated. Organization, copy number, and methylation pattern of the FBP gene

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‡ Abbreviations: FBP, folate-binding protein; SAM, *S*-adenosylmethionine; KB-FD, KB cells grown in folate-deficient medium containing less than 10 nM folate; KB-FR, KB cells grown in standard folate-replete medium containing at least 2 μM folate; AzaC, 5-azacytidine; ODC, ornithine decarboxylase; and SSC, standard saline citrate (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0).

in KB-FR and KB-FD were examined. The transcriptional rate of the FBP gene and FBP mRNA stability were also compared between KB-FR and KB-FD. These results indicate that increases in FBP and FBP mRNA are accomplished, at least in part, through regulating FBP mRNA stability.

MATERIALS AND METHODS

Materials. [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]UTP (3000 Ci/mmol) were obtained from DuPont/New England Nuclear (Boston, MA). Actinomycin D and 5-azacytidine (AzaC) were obtained from the Sigma Chemical Co. (St. Louis, MO). Minimum essential medium without folic acid and fetal bovine serum were obtained from GIBCO BRL (Gaithersburg, MD). Restriction endonucleases were obtained from United States Biochemical (Cleveland, OH). All other reagents were of the highest quality available.

Cell culture. Human KB cells (ATCC CCL 17) were obtained from the American Type Culture Collection (Rockville, MD), and were propagated continuously in 10% fetal bovine serum supplemented minimum essential medium with 2.3 μM folic acid (KB-FR) or without any folic acid (KB-FD) at 37° in a humidified atmosphere of 5% CO_2 for more than 6 months. The 10% fetal bovine serum provides the only source of folate (approximately 2–10 nM) in the growth medium of KB-FD [3, 18]. So far, only one type of FBP (putative adult form) appears to be present in KB cells [7, 8]. We observed, as other groups have reported [3, 7, 18, 20], an approximately 3- to 5-fold increase of FBP mRNA and FBP in KB-FD compared with KB-FR. The increase of FBP mRNA in KB-FD was due to the increase in the adult species as determined by a quantitative polymerase chain reaction assay*. The doubling times of KB-FR and KB-FD were found to be approximately 22 and 28 hr, respectively. For AzaC treatment, KB-FR cells were exposed to 5 μM AzaC by drug addition 24 hr after each passage (approximately 20–30% confluent) repeatedly for 3 weeks. All the other experiments were performed when cells were grown to confluence. Cells were checked regularly for Mycoplasma contamination and consistently tested negative.

Plasmids and probes. pG4ZF511, a gift from Dr. Stephen W. Lacey (University of Texas Southwestern Medical Center, Dallas, TX), contains a 991 bp *Eco*RI cDNA fragment prepared from human Caco-2 (colon carcinoma) FBP mRNA [1]. pODC10/2H, a human ornithine decarboxylase (ODC) cDNA clone [22], was a gift from Dr. Ajit K. Verma (University of Wisconsin, Madison, WI). The 544 bp human β-actin cDNA fragment, spanning positions −9 to 535 of the mRNA sequence [23], was generated from KB RNA by using a β-actin mRNA sequence-specific complementary primer in a reverse transcription reaction and further amplification of the resulting cDNA by the polymerase chain reaction [24]. For northern and Southern blot analyses, the gel-purified double-stranded cDNA fragments from restricted plasmids (pG4ZF511 and pODC10/2H) and β-actin PCR products were used in preparing ^{32}P-labeled probes by the random priming method [25]. p11/14 containing a chicken β-actin cDNA was a gift from Dr. Joseph T. Y. Lau (Roswell Park Cancer Institute, Buffalo, NY) [26]. pG4ZF511, p11/14 and pGEM-1 (Promega, Madison, WI) were used as DNA probes in filter hybridization for nuclear run-off transcription assay.

Preparations and analyses of nucleic acids. Total cellular RNA was isolated from cultured cells using guanidine thiocyanate according to the method of Chirgwin *et al.* [27]. Northern blot analysis, prehybridization and hybridization of filters, and genomic DNA isolation were carried out as previously described [28]. Restriction enzyme digestion of DNA and Southern blot analysis were performed as described by Sambrook *et al.* [29]. Filters were prehybridized at 42° for 12 hr, and then hybridized to ^{32}P-labeled DNA probes of specific activity greater than 10^9 cpm/μg at 42° for 12–16 hr. Unbound probes were removed by washing filters with two changes of a solution containing 2× standard saline citrate (SSC) and 0.2% sodium dodecyl sulfate (SDS) for 30 min each at room temperature, and two changes for 30 min each of 0.2× SSC and 0.2% SDS at 60°. Blots were exposed to Kodak X-OMAT AR film with intensifying screens, and specific bands were further quantitated by radioimaging using a Molecular Dynamics PhosphorImager.

Nuclear run-off transcription assay. Nuclei were isolated and further purified over a sucrose cushion by published methods [30, 31]. Nuclear run-off transcription assays were performed with 2×10^7 nuclei based upon the method described by Celano *et al.* [32] except that 450 μCi [α-^{32}P]UTP and 0.6 mM ATP, GTP and CTP were used. The amount of [α-^{32}P]UTP incorporated into nuclear RNA was determined by the trichloroacetic acid precipitation method [29], and equal amounts of ^{32}P-labeled nuclear RNA from KB-FR and KB-FD were used during filter hybridizations. Immobilization of DNA probes onto nylon filters was performed according to the method of Kafatos *et al.* [33]. Five micrograms of denatured plasmid DNA was dot-blotted to nylon filters using a 96-well filtration manifold with 3-mm wells. The filters were UV irradiated and prehybridized at 50° for 12–16 hr. ^{32}P-Labeled nuclear RNA from each nuclear run-off reaction (usually more than 10^6 cpm) was incubated in 0.5 mL of hybridization buffer at 55° for 15 min, and then combined with a filter containing the β-actin cDNA, FBP cDNA, and pGEM-1 vector at 50° for 72 hr. Filter-washing conditions, and prehybridization and hybridization buffers used were the same as those used in the northern and Southern blot analyses, except for an additional wash with 2× SSC containing RNase A (20 μg/mL) at room temperature for 30 min. Autoradiography and radioimaging analysis were performed as described above.

mRNA half-life studies. KB-FR and KB-FD cells were exposed to 5 μg/mL actinomycin D as previously described [34]. Total cellular RNA from drug-treated cells was isolated at various times, and analyzed by northern blotting with ^{32}P-labeled FBP, ODC and

* Hsueh C-T and Dolnick BJ, unpublished observation.

β-actin cDNA probes. For the determination of equal gel loading, RNA samples were treated with ethidium bromide prior to loading [35], and visualized in the formaldehyde agarose gels under UV illumination after the electrophoresis was complete (photograph not shown).

Statistical analysis. All experiments were performed at least twice with similar results, and the results of one representative experiment are reported. The curve-fitting analysis in mRNA half-life studies was performed by linear regression.

RESULTS

Southern blot analysis of the FBP gene. It has been shown that folate-deficient conditions *in vitro* and *in vivo* are associated with a variety of chromosomal abnormalities such as chromosome breaks and gaps, increased sister chromatid exchanges, and incomplete chromosome contraction [36, 37]. Recently, Brigle *et al.* [10] reported that enhanced expression of one FBP mRNA in murine L1210 leukemia cells grown in folate-deficient medium might be a result of gene rearrangement because a shorter RNA species and altered restriction patterns (e.g. *Hin*dIII) of the FBP gene were observed. These results prompted us to compare the general organization of the FBP gene in KB-FR and KB-FD by Southern blot analysis. DNAs from KB-FR and KB-FD cells were digested with restriction endonucleases, fractionated by agarose gel electrophoresis, and blotted to nylon filters. Using ^{32}P-labeled FBP cDNA (putative adult form) as a probe, we observed no differences in the *Hin*dIII-restriction pattern between KB-FR and KB-FD (Fig. 1A) or in restriction patterns generated by digestion of DNA with *Apa*I, *Ban*II, *Bgl*I, *Bgl*II, *Eco*RI, *Pst*I, *Sal*I, *Sma*I, or *Xho*I (data not shown). This indicates there was no major alteration in the genomic FBP locus in KB-FD. Southern blot analysis also showed no detectable increase in the FBP gene dosage in KB-FD (Fig. 1A, as determined by radioimaging). Since there is high degree of similarity among adult FBP cDNA, fetal FBP cDNA and pseudogene(s) [9, 12, 13], it is likely that not only the adult gene but also the fetal gene and pseudogene(s) could be detected by Southern blot analysis in this study.

Methylation patterns of the FBP gene. Methylation of DNA at cytosine residues of certain CpG sites is mediated by DNA methyltransferase using SAM as the methyl group donor [38, 39]. In some instances, a decrease in methylation is associated with gene activation and an increase in methylation is associated with gene inactivation, implying that the methylated cytosine residues of CpG dinucleotides are involved in transcriptional control of gene expression [40, 41].

To compare the methylation patterns of the FBP gene between KB-FR and KB-FD, Southern blot analysis was performed. *Msp*I- and *Hpa*II-restricted genomic DNAs were probed with ^{32}P-labeled FBP cDNA. *Msp*I and *Hpa*II are isoschizomer restriction-enzymes that recognize the sequence CCGG. *Msp*I does not cleave DNA when the external cytosine is methylated and is not sensitive to internal cytosine methylation, whereas *Hpa*II does not digest DNA if the internal cytosine is methylated [42]. There was no difference in the *Msp*I-FBP gene restriction pattern from KB-FR or KB-FD (Fig. 1B), but there were three DNA fragments (approximately 11, 6 and 5 kbp in size) that were not evident in the *Hpa*II-restricted FBP gene of KB-FD (Fig. 1C). These three DNA fragments were also less apparent in the *Hpa*II-restricted FBP gene from KB-FR exposed to 5 μM AzaC for 3 weeks (Fig. 1C), but AzaC treatment of KB-FR had no effect upon the *Msp*I-restriction pattern (Fig. 1B). AzaC has been shown to replace cytidine residues in replicating DNA and prevent methylation, thereby inducing hypomethylation of DNA [40]. The replacement of AzaC on the internal C of CCGG sequences raised the susceptibility of the FBP gene to *Hpa*II restriction in AzaC-treated KB-FR; hence, the *Hpa*II-restricted FBP genomic fragments exhibited smaller sizes and some of them less hybridization intensity on the Southern blot, compared with KB-FR (Fig. 1C). These results indicate hypomethylation of some internal CCGG cytosine residues in the FBP gene in KB-FD or KB-FR grown in the presence of AzaC.

Effect of DNA methylation inhibitors on the steady-state levels of FBP mRNA in KB-FR. Hypomethylation of the FBP gene in KB-FD suggested the possibility of inducing FBP gene expression in KB-FR by exposing cells to the DNA methylation inhibitor AzaC. Northern blot analysis of RNAs extracted from KB-FR grown in the absence or presence of 5 μM Azac for 3 weeks revealed no increase of the FBP mRNA levels in the AzaC-treated KB-FR (Fig. 2). However, we did observe decreased FBP mRNA levels in AzaC-treated KB-FR. This might be due to the RNA incorporation effect of AzaC, which may affect the synthesis and processing of RNA [40, 43]. There was no induction of FBP mRNA levels when KB-FR cells were exposed to AzaC for 72 hr, or to another DNA methylation inhibitor, 5-aza-2′-deoxycytidine [40], for 72 hr or 3 weeks (data not shown). These results imply that the induction of FBP mRNA expression in KB-FR may require selective hypomethylation within the FBP locus and/or some other genes instead of a generalized DNA hypomethylation (as would be induced by the DNA methylation inhibitors), or that the hypomethylation changes associated with the FBP gene in KB-FD may not be related to the enhanced FBP mRNA expression.

Transcriptional rates of the FBP gene. To analyze further the mechanism for increased FBP mRNA levels in KB-FD, nuclear run-off transcription assays were performed. Nuclei from KB-FR and KB-FD were isolated, and the amounts of initiated RNA transcription were measured for β-actin and FBP by filter hybridization and radioimaging. After normalization to the transcriptional rates of the β-actin gene, the transcriptional rates of the FBP gene were found to be approximately the same in KB-FR and KB-FD as is evident from visual inspection of the autoradiogram (Fig. 3).

FBP mRNA stability. mRNA half-life studies were carried out to determine whether enhanced mRNA stability could account for the increase in the steady-state levels of FBP mRNA in KB-FD. KB-FR and KB-FD were exposed to 5 μg/mL of the transcription inhibitor actinomycin D [44]. At various times total RNA was isolated, and northern blot analysis was

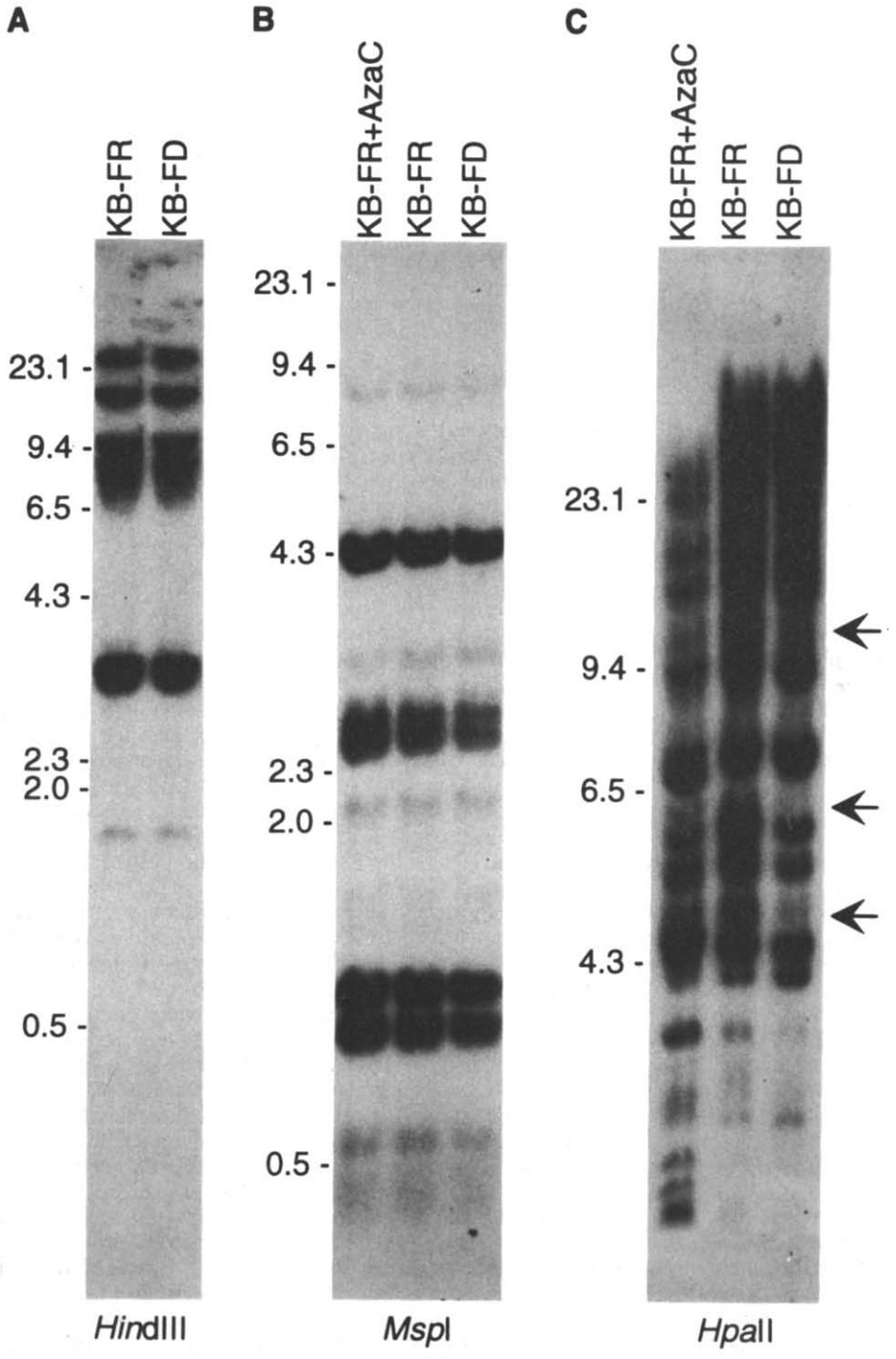


Fig. 1. Restriction analyses and methylation patterns of the FBP gene. Genomic DNA (15 μg) was digested with the indicated restriction endonucleases, fractionated by electrophoresis through 0.6% agarose gels, blotted to nylon filters, and then hybridized with ^{32}P-labeled FBP cDNA (the *Eco*RI insert of pG4ZF511). The positions of *Hin*dIII-digested λ phage DNA molecular weight markers are indicated to the left in kilobase pairs. (A) *Hin*dIII-restricted pattern of the FBP gene in KB-FR and KB-FD DNA. (B) *Msp*I-restricted pattern of the FBP gene in KB-FR exposed to 5 μM AzaC for 3 weeks, KB-FR and KB-FD. (C) *Hpa*II-restricted pattern of the FBP gene in KB-FR exposed to 5 μM AzaC for 3 weeks, KB-FR and KB-FD. Arrows to the right indicate the positions of three *Hpa*II-restriction fragments that were present in KB-FR DNA but not evident in KB-FD DNA.

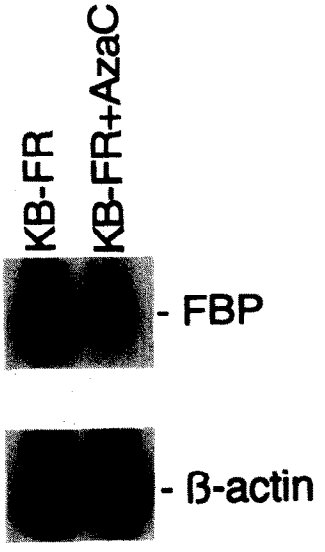


Fig. 2. Steady-state levels of FBP mRNA in AzaC-treated KB-FR. Total cellular RNA was extracted from KB-FR grown in the absence or presence of 5 μM AzaC for 3 weeks, and northern blot analysis was performed with 10 μg of RNA/lane. After electrophoresis, the RNAs were blotted to nylon filters, which were sequentially hybridized to ^{32}P-labeled FBP and β-actin cDNAs.

performed with ^{32}P-labeled ODC, FBP and β-actin cDNA probes (Fig. 4A). There were no substantial differences between KB-FR and KB-FD in the half-lives of ODC mRNA (KB-FR 7.7 hr, KB-FD 9 hr) or β-actin mRNA (KB-FR 9.2 hr, KB-FD 11.8 hr) within the time frame studied, and these decay rates were consistent with reported values [45, 46]. The half-life of FBP mRNA was determined to be approximately 5.7 hr in KB-FR and 14 hr in KB-FD (Fig. 4B). The FBP mRNA half-life increased 2.5-fold in KB-FD, while the steady-state levels of FBP mRNA increased approximately 5-fold (Fig. 4A).

DISCUSSION

Brigle *et al.* [10, 47] have shown that in murine L1210 leukemia cells adapted to long-term culture in folate-deficient medium, enhanced FBP gene expression might be due to the insertion of a retrovirus-like sequence into the 5′-regulatory region of the FBP gene. In the present study, we observed no difference between KB-FR and KB-FD in the major organization of the FBP gene, and in the size of FBP mRNA (Fig. 4A). Therefore, the mechanism for enhanced FBP gene expression in KB-FD is likely different from that of murine L1210 cells grown in folate-deficient medium.

It has been shown that the intracellular folate pool in KB-FD decreases 20-fold after 1 month of growth in folate-deficient medium [48], but the increase in FBP usually plateaus in 2–3 months [3, 18, 20]. Since the change of FBP gene expression associated with folate depletion has been known to be relatively gradual and slow, it led us to speculate that enhanced FBP gene expression might be related to alterations in DNA methylation, which could be affected by changes in folate-dependent biochemical reactions. Methylation of DNA at cytosine residues of certain CpG sites is mediated by DNA methyltransferase using SAM as the methyl group donor [38, 39]. Methyl-deficient states *in vivo*, including folate deficiency, can lead to decreased levels of SAM [39], and result in hypomethylation of DNA [49, 50]. A preliminary report by Cravo *et al.* [51] has indicated that folate deficiency in rats causes hypomethylation in their liver DNA. Hypomethylation changes in the FBP gene of KB-FD were observed in the present study, and these changes could also be induced by exposing KB-FR to a DNA methylation inhibitor (Fig. 1C). Because the sequence of the FBP gene is still not known, we have not been able to identify at which sites hypomethylation of the FBP gene occurred in KB-FD. Whether other genes were hypomethylated in KB-FD was not investigated.

While growing KB-FD in folate-replete medium for over 3 months, we also observed that one of these three hypomethylated DNA fragments (11 kbp)

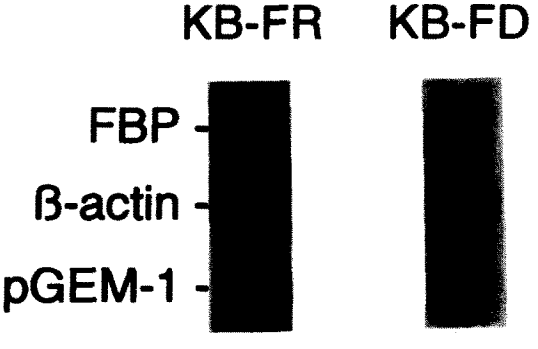
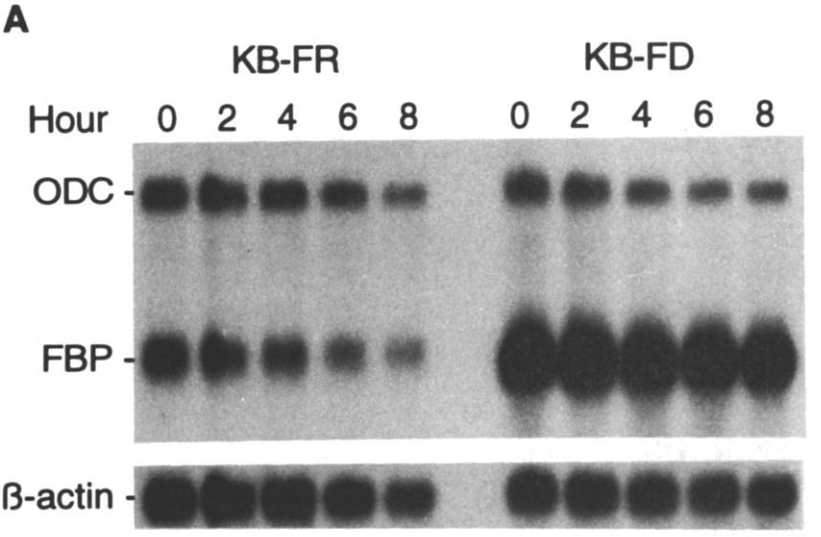


Fig. 3. Transcriptional rates of FBP gene in KB-FR and KB-FD. Nuclei (2×10^7) from KB-FR and KB-FD were used in nuclear run-off transcription assays as described under Materials and Methods. Equal amounts of ^{32}P-labeled nuclear RNA were hybridized to filters containing 5 μg of denatured plasmids containing cDNAs for β-actin and FBP genes. Denatured pGEM-1 plasmid DNA (5 μg) was used as a negative control.



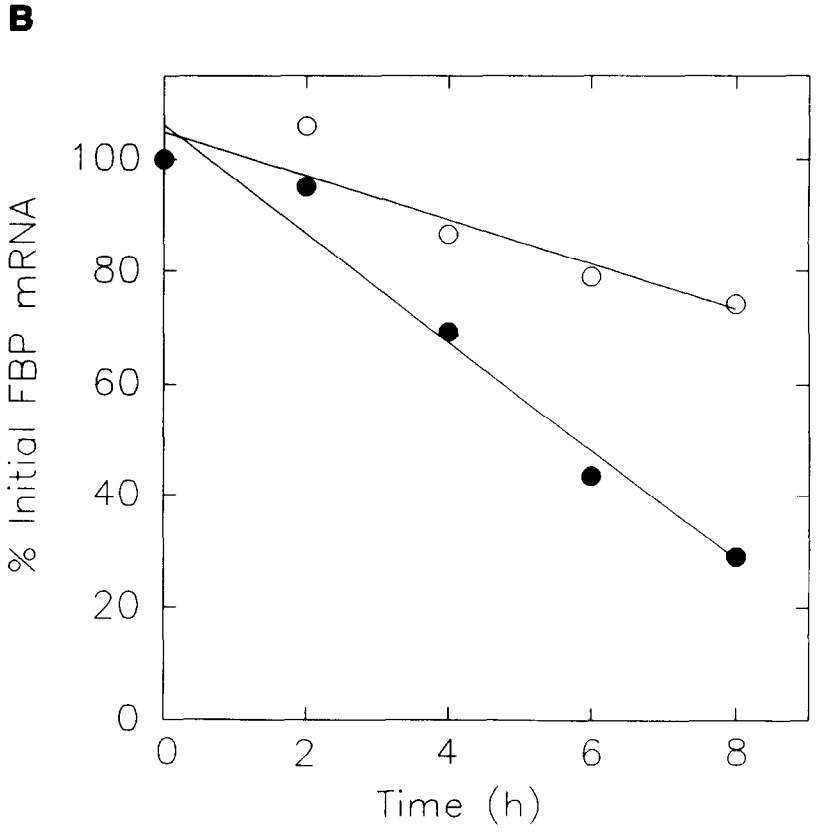


Fig. 4. Half-life studies of ODC, FBP and β-actin mRNAs from KB-FR and KB-FD. (A) Northern blot analysis. KB-FR and KB-FD were exposed to actinomycin D (5 μg/mL) for the indicated times. Total cellular RNA (10 μg) was analyzed as described. (B) Half-life determination of FBP mRNA. Specific FBP mRNA bands for KB-FR (●) and KB-FD (○) in the RNA blots were quantitated by radioimaging. The lines represent the results of best fit analysis determined by linear regression.

was again methylated, and the steady-state levels of FBP and FBP mRNA returned to the baseline levels found in KB-FR*. Our observation, along with another report [18], suggests that FBP gene expression in KB cells is regulated by the extracellular folate concentration through changes in intracellular folate levels. This result is in contrast to FBP gene expression in murine L1210 leukemia cells, which is not regulated by the folate status of the cell [47].

DNA methylation plays an important role in the transcriptional control of many eukaryotic genes such as human globin and rat growth hormone [41, 52]. In recent years, several methyl-CpG binding proteins have been identified, and it has been hypothesized that these proteins could complex with methylated CpG dinucleotides in the promoter region and prevent the access of transcription factors [53, 54]. Therefore, the hypomethylation changes of the FBP gene in KB-FD suggested the possibility of inducing FBP gene expression in KB-FR by DNA methylation inhibitors. However, the DNA methylation inhibitors failed to induce FBP mRNA expression in KB-FR. Additionally, there was no detectable difference in the transcriptional rate of the FBP gene between KB-FR and KB-FD. These findings suggest that hypomethylation of the FBP gene in KB-FD is probably not involved in a *cis*-related manner to changes that lead to the elevated FBP and FBP mRNA levels found in KB-FD, and that the FBP gene in KB-FD may be regulated post-transcriptionally.

Previously, we observed that KB1BT cells, a methotrexate-resistant KB line, had decreased FBP levels compared with KB cells, and the decrease in FBP levels correlated well with the decrease in FBP mRNA levels [55]. We also observed that both FBP and FBP mRNA increased approximately in the same time frame and to the same degree during the adaptation of KB cells to growth in folate-deficient medium.† These findings suggest that the regulation of FBP gene expression possibly occurs proximal to translation. mRNA half-life studies presented here have shown that the enhanced stability of FBP mRNA could account for half of the observed increase in the steady-state levels of FBP mRNA in KB-FD. Whether other factors such as nuclear to cytoplasmic transport of mRNA, or pre-mRNA processing may be involved in the post-transcriptional regulation of increased FBP mRNA levels in KB-FD requires further investigation.

Regulation of mRNA stability has been implicated in controlling the gene expression of certain proteins and oncogenes [56]. Many lines of evidence have shown there are both *cis*-acting elements, such as the secondary structure of mRNA or specific mRNA sequences, and *trans*-acting factors, such as ribonucleases or RNA-binding proteins, involved in regulating mRNA stability [57]. The identification of potential *cis*- and *trans*-acting factors involved in controlling FBP mRNA life span in KB-FD will be informative in the regulation of FBP gene expression.

In summary, our data indicate that there is no difference between KB-FR and KB-FD in the major genomic organization, copy number or transcriptional rate of the FBP gene. The hypomethylation of the FBP gene accompanying the increased FBP mRNA levels could not account for the increased FBP gene expression in KB-FD, but the increased FBP mRNA stability is partly responsible for the increased FBP gene expression in KB-FD.

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