Enhancement of energy metabolism is fundamental to the developmental programs of many cell types. This work examines the molecular mechanisms that mediate changes in energy metabolism during differentiation of osteoblastic cells. When the rat osteoblastic cell line, ROS 17/2.8, is induced to differentiate with 1,25-dihydroxyvitamin D₃, expression of creatine kinase-b (ck-b), a pivotal enzyme in energy metabolism, is enhanced. Maximum enhancement occurs at 48 h of induction with 10 nm 1,25-dihydroxyvitamin D₃ when creatine kinase activity is 2.1-fold over undifferentiated cells. This is associated with a 2-fold increase in transcription rate and the formation of a second protein-DNA complex on the ck-b gene promoter that is supplementary to the one present in undifferentiated cells. In addition, the contribution of posttranscriptional regulatory mechanisms is suggested by (1) the increase in ck-b mRNA abundance exceeds that of transcription rate, indicating an increase in message stability, (2) the increase in ck-b mRNA precedes and exceeds that of protein activity, indicating translational modulation, and (3) RNA mobility-shift assays indicate that a cytosolic factor in ROS 17/2.8 cells interacts specifically with the highly conserved 3'-untranslated region of the ck-b mRNA. We have previously reported that such an interaction mediates translational control (Ch'ng, J. L. C., Shoemaker, D. L., Schimmel, P., and Holmes, E. W. (1990) Science 248, 1003-1006). The physiological roles of these regulatory mechanisms during osteoblast differentiation are discussed.

Enhancement of energy metabolism is an integral component of differentiation in many cell systems. During differentiation of osteoblasts, energy metabolism is up-regulated to meet increased energy demands for functions such as changes in cell shape and enhanced activity of membrane pumps. Changes in cell shape facilitate adherence to bone surfaces and serve the purpose of limiting exposure of bone to osteoclasts (1). Enhanced activity of membrane pumps is related to the formation of extracellular matrix and mineralization. This increase in energy metabolism is represented by an increase in the specific activity of creatine kinase-b (ck-b), a pivotal enzyme in cellular energy metabolism in the osteoblast (2, 3). Creatine kinase plays a central role in cellular energy metabolism by catalyzing the reversible transfer of high energy phosphate bonds from creatine phosphate to ADP to regenerate ATP (4). Hence, it acts as a transducer of high energy phosphate bonds from sites of production to sites of utilization. This ensures that there is no depletion of ATP in subcellular components where energy is being rapidly expanded. The importance of creatine kinase in energy metabolism is seen in the stringent regulation of its expression, both developmentally and spatially. Creatine kinase activity has been shown to be closely associated with the cytoskeleton, providing ATP for microtubular movement (5). Cytoskeletal changes and alteration in cell shape occur in osteoblasts which have been induced to differentiate with 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) (6). Shapiro and colleagues (7) have demonstrated that the level of ck-b activity is directly related to chondrocyte maturation and a decrease in enzyme activity occurs in vitamin D deficiency. Similar findings were reported by Katoh and colleagues (8). Studies in rats indicate that creatine kinase activity is necessary for proper matrix maturation and mineral deposition (9). Inhibition of enzyme activity by administering β-guanidinopropionic acid results in profound changes including disorganization of chondrocytes in the proliferative and hypertrophic zones, poor vascularization, changes in the electrophoretic mobility of type II and type X collagens and inhibition of apatite formation. Hence, enhancement of ck-b activity is necessary for normal development of bone.

The molecular mechanisms that mediate the up-regulation of energy metabolism during osteoblast differentiation are largely unknown. By studying the regulation of ck-b, a pivotal enzyme in cellular energy metabolism in osteoblasts, insight could be derived on these modulatory mechanisms as well as on differentiation of osteoblasts. For instance, during muscle differentiation, expression of creatine kinase-m, the predominant cytosolic isoenzyme of creatine kinase in muscle, is markedly increased (10, 11). This is mediated by the interaction of MyoD, an essential factor in muscle differentiation, with the promoter of the ck-m gene (12). A similar mechanism may occur during osteoblast differentiation involving the interaction of a transcriptional activator with one of the many positive-acting elements in the ck-b gene promoter (13, 14). The known positive-acting elements are mainly 3' of -195 nucleotides (nt) from the transcription start site (15, 16). It has been shown that two different RNA polymerase II initiation complexes can assemble on the rat ck-b promoter (17). There are several elements that show homology to the responsive elements of known transcription factors including those for MyoD (E-box motif), adenovirus E1a protein, Sp1, and CTF-1 (13). An AT-rich sequence at -60 binds the TA-rich recognition protein (TARP) which is found in several tissues and is likely to be a positive-acting transcription factor for the ck-b gene (18). However, it is not developmentally regulated, and thus far, no element has been
described that is differentiation-specific. Further, there is no recognizable vitamin D-responsive element.

It was demonstrated that translational regulation of ck-b is significant in monocytes (19). This is mediated by the 3'-untranslated region of the ck-b mRNA which is highly conserved across species (>70% for chicken:man) (20). This region of the ck-b mRNA interacts with a cytosolic factor to form a protein-RNA complex at the 3' end of the message. Translation is inhibited when the complex forms; conversely, translation is enhanced when affinity of the cytosolic factor for the cis-acting RNA element decreases, resulting in dissociation of the complex.

This study shows that ck-b is specifically up-regulated during differentiation of osteoblastic cells. This is mediated by an increase in transcription rate and is associated with the formation of an additional protein-DNA complex on the ck-b gene promoter. Evidence is also presented on the interaction of a cytosolic factor in osteoblastic cells with the highly conserved 3'-untranslated region of the ck-b mRNA. It is hypothesized that these mechanisms facilitate the enhancement of ck-b expression as well as its targeting to high energy-expanding subcellular structures in the mature osteoblast.

**MATERIALS AND METHODS**

**ROS 17/2.8 Cells**—ROS 17/2.8 cells were a generous gift from Dr. G. Rodan (Merk Sharp & Dohme Research Laboratories, West Point, PA). This rat cell line was derived from an osteosarcoma and retained many features of osteoblasts (21). Cells were plated on 10-mm Petri dishes and cultured in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10% charcoal-stripped fetal calf serum. Incubation with 10 nM 1,25-(OH)2D3 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) was started when cells were ~20% confluent. Control cells were incubated with a similar concentration of vehicle (ethanol). The concentration of ethanol in treated and control cells did not exceed 0.1%. Cells were harvested for analysis while in log phase growing (<70% confluent).

**ck-b-specific Activity Assay**—A sensitive radiolabelled assay was used to measure the specific activity of ck-b (22). We have previously demonstrated that creatine kinase activity measured by this assay parallels creatine kinase protein abundance as measured by Western blotting but is more sensitive (23). The assay measures the creatine kinase-catalyzed conversion of 44C-labeled creatine to creatine phosphate. Labeled creatine phosphate is separated from unlabeled creatine by binding to charged DEAE disks. Formation of creatine phosphate is favored by coupling the reaction to the pyruvate kinase-catalyzed conversion of ADP to ATP in the presence of phosphoenolpyruvate. This assay for creatine kinase activity is highly specific and has a sensitivity of <0.5 milliunits/mg of protein when using 14Ccreatine with a specific activity of >15 Ci/mM (Amersham Corp.).

To assay for creatine kinase activity, ROS 17/2.8 cells were trypsinized and pelleted by centrifugation. They were then washed with phosphate-buffered saline, re-centrifuged, and lyzed in 50 mM Tris (pH 8.8) and 1 mM dithiothreitol by three cycles of freeze-thawing in an ethanol-dry ice mixture. Soluble cytosolic extract was separated from insoluble cellular components by centrifugation and stored at 20 °C until assay. Ten micrograms of protein extract, as measured by Bio-Rad protein assay, were added to each reaction mixture to assay for creatine kinase activity.

**Creatine Kinase-b mRNA Abundance**—ROS 17/2.8 cells were incubated with 10 nM 1,25-(OH)2D3 or vehicle in 10-cm Petri dishes. At timed intervals, total RNA was isolated from the cells with guanidinium thiocyanate and purified by centrifugation through a cesium chloride gradient. ck-b mRNA abundance was assayed by Northern blot analysis (24). Fifteen micrograms of total RNA was electrophoresed on a 1% denaturing formaldehyde-agarose gel and transferred overnight onto a Nitran membrane. The RNA was cross-linked to the membrane by a StrataGene UV Stratalinker. The membrane was hybridized with a 32P-labeled probe made by random priming with rat ck-b cDNA as the template (Multiprime DNA Labeling System, Amersham Corp.). Rat ck-b cDNA was a gift from Dr. Pamela Benfield (E. I. Du Pont, Wilmington, DE). Following overnight hybridization at 42 °C, the Northern blot was washed at high stringency (65 °C, 0.1 x SSC) before autoradiography. ck-b mRNA gives a signal at 1.3 kb in size. As control, the filter was simultaneously hybridized to a similarly labeled 1.25-(OH)2D3-treated ROS 17/2.8 cells was determined by nuclear run-on analysis (26). Approximately 2 x 107 cells were removed from Petri dishes by scraping with a Teflon scraper. The cells were pelleted by centrifugation and lysed in 4 ml of Nonidet-40 lysis buffer. Nuclear were pelleted by centrifugation at 500 x g at 4 °C, resuspended in 4 ml of Nonidet-40 lysis buffer, vortexed, and centrifuged again under similar conditions. The nuclear pellet was then resuspended in 200 ml of glyceral storage buffer and stored in liquid nitrogen until assayed.

To perform the run-on assay, nuclei were thawed and incubated with 50 mM ATP, CTP, and GTP, and 100 μg of [3H]UTP (800 Ci/mM, Amersham Corp.) at 30 °C, with shaking, for 30 min. RNase-free DNase I and proteinase K (Sigma) were added, phenol/chloroform extraction was performed twice, and labeled RNA was precipitated with ethanol. This was then hybridized to a Nytran membrane to which 25 μg of rat ck-b cDNA and β-actin cDNA (internal standard) had been cross-linked. Hybridization was done at 55 °C in 50% formamide for 16 h. The filter was washed at high stringency with the last wash being 0.1 x SSC at 65 °C, followed by incubation with 10 μg/ml RNase A in 0.3 x NaCl at 37 °C for 10 min. The membrane was autoradiographed, and the intensity of the ck-b signal (measured by laser densitometry) relative to that of β-actin was compared between control and 1,25-(OH)2D3-treated cells.

**DNA-Nuclear Factor Interaction**—DNA-nuclear factor interactions were studied by DNA mobility-shift assay using gel electrophoresis (17). By restriction enzyme digestion, a 421-bp DNA fragment, that spans from -192 to +220 nt relative to the transcription start site of the ck-b gene, was obtained. This fragment, which includes a sequence 3' to the start site, contains all of the elements reported to be essential for ck-b gene expression (15, 16). It was end-labeled with 32P and incubated with nuclear extracts from control and 1,25-(OH)2D3-treated (10 nM for 48 h) cells. Nuclear extracts were obtained using the protocol of Dignam et al. (27). Briefly, cells were seeded onto a Douche homogenizer, nuclei were precipitated by centrifugation, lysed in high salt buffer and rinsed by dialysis at 4 °C for 6 h. Each reaction mixture (total volume = 20 μl) contained 5 μg of nuclear extract and 0.2 ng of labeled DNA sequence (~105 cpm). To reduce nonspecific interactions, poly(dI-dC)-poly(dI-dC) (Boehringer Mannheim) and bovine serum albumin were added. The mixture was incubated at 30 °C for 15 min before being applied to a 4% non-denaturing, low ionic strength polyacrylamide gel and electrophoresed at 200 V in low ionic-strength buffer. The gel was dried and autoradiographed.

**RNA Mobility-shift Assay**—Specific interactions between cytosolic factors and the ck-b mRNA were analyzed by RNA mobility-shift assays (19). RNA probes corresponding to the entire 3'-untranslated region (195 bases) and adjacent 3'-coding region of the ck-b mRNA (65 bases) were transcribed from the pBlueScribe in-vitro system (Stratagene Cloning Systems, San Diego, CA) with T7 RNA polymerase. The probes were labeled with [32P]UTP and purified by denaturing polyacrylamide gel electrophoresis. Twenty nanomoles of RNA probe (107 cpm) were incubated with 40 μg of S100 (cytosolic) extract from control and 1,25-(OH)2D3-treated (10 nM for 48 h) cells. Each reaction mixture was incubated at 30 °C, with shaking, for 30 min and then precipitated by centrifugation, lysed in high salt buffer and rinsed by dialysis at 4 °C for 6 h. Each reaction mixture total volume = 20 μl) contained 5 μg of nuclear extract and 0.2 ng of labeled DNA sequence (~105 cpm). To reduce nonspecific interactions, poly(dI-dC)-poly(dI-dC) (Boehringer Mannheim) and bovine serum albumin were added. The mixture was incubated at 30 °C for 15 min before being applied to a 4% non-denaturing, low ionic strength polyacrylamide gel and electrophoresed at 200 V in low ionic-strength buffer. The gel was dried and autoradiographed.

**RESULTS**

**Enhancement of ck-b Expression by 1,25-(OH)2D3**—Three separate experiments were done to study time course effects with 10 nM 1,25-(OH)2D3. There was a significant change in ck-b activity at 24 h of 1,25-(OH)2D3 incubation with an increase of 1.4-fold over control. The increase peaks at 48 h with a 2.1-fold enhancement and remains at this level for the remainder of the time course (96 h) (Fig. 1). Ten nM 1,25-(OH)2D3 was used in these studies as this is the concentration used in similar studies reported in the literature and our preliminary studies have shown this to be the most effective concentration for...
activity in ROS

message abundance of ck-b in ROS 17/23 cells is unaffected by experiments were done to study changes in mRNA abundance. mRNA abundance was normalized to that of p-actin. Harrison determined by Northern analysis and the quantitation of ck-b at various time points of 1,25-(OH)₂D₃ treatment with the intensity of ethidium bromide staining for 28 S and 18 S ribosomal RNA (data not shown). A 1.3-fold increase in ck-b mRNA at 48 h and is maintained for the duration of the study (96 h). Hence, the increase in ck-b mRNA abundance preceded and occurs following differentiation of ROS 17/2.8 cells. However, there is no positive feedback mediated by 1,25-(OH)₂D₃ on the expression of its receptor is functional in these cells. But, there is no element in the ck-b gene promoter that resembles the cognate sequence for the vitamin D receptor. This suggests that the effect of 1,25-(OH)₂D₃ on ck-b expression in ROS 17/2.8 cells is indirect and is consequent to its induction of differentiation.

Transcription rate of ck-b—Transcription rate of ck-b in ROS 17/2.8 cells was measured by nuclear run-on experiments. An internal standard, β-actin, was used for both control and treated cells. Transcription of β-actin, as determined by nuclear run-on assay, is unaltered by 1,25-(OH)₂D₃ in ROS 17/2.8 cells (25). Transcription of ck-b in cells treated for 48 h with 10 nm 1,25-(OH)₂D₃ is increased 2-fold (Fig. 3). As ck-b mRNA abundance peaks at 48 h and remains at this level for up to 96 h, it is likely that the transcription rate at 48 h is the peak level induced by 1,25-(OH)₂D₃. Hence, there is a significant difference between the increase in transcription rate and the increase in mRNA abundance at 48 h of incubation with 1,25-(OH)₂D₃, the increases being 2- and 5.7-fold, respectively. This suggests that, in addition to transcriptional enhancement, a second mechanism, involving increased ck-b message stability, occurs following differentiation of ROS 17/2.8 cells.

Nuclear Protein-ck-b Promoter Interactions during Differentiation of ROS 17/2.8 Cells—This was studied by DNA mobility-shift assay using a labeled 421-bp DNA fragment that corresponds in sequence from -192 to +228 nt relative to the transcription start site of the ck-b gene promoter. It has previously been shown that this fragment contains all known positive-acting promoter elements (15, 16). The data are shown in Fig. 4. Lanes 1, 2, and 3 contain nuclear extracts from ROS 17/2.8 cells treated with 10 nm 1,25-(OH)₂D₃ for 48 h. Lanes 4 and 5 contain extracts from control cells. Lanes 1 shows that two (an upper and a lower) nuclear factor-DNA complexes are formed with nuclear extracts from 1,25-(OH)₂D₃-treated cells. Both complexes are competed out when >100-fold excess of unlabeled DNA corresponding to the entire 421 bp is added in lane 3 with a >100-fold excess of an unlabeled 228-bp fragment that corresponds in sequence to +1 to +228 nt. Only the upper complex is present when the labelled promoter is incubated with nuclear extracts from control cells in lane 4. The addition of 1,25-(OH)₂D₃ in vitro to nuclear extracts from control cells did not reproduce the lower complex in lane 5. These interactions are specific as (a) poly(dl-dC)-poly(dl-dC) and bovine serum albumin are added to hinder nonspecific interactions and (b) both complexes are susceptible to in vitro competition with unlabeled DNA that is similar in sequence as the promoter. Competition studies with unlabeled DNA fragments indicate that the cis-acting element for the constitutive complex is within -192 to +1 nt while that for the differentiation-induced complex is within +1 to +228 nt.

These studies demonstrate that the enhancement of ck-b transcription during differentiation is associated with the formation of an additional protein-DNA complex (lower band on gel shift assay) on the ck-b gene promoter. The upper complex which is present in both the control and differentiated cells could be associated with constitutive expression. 1,25-(OH)₂D₃ itself does not appear to be a component of the lower complex as its addition in vitro to the reaction mixture containing nuclear extracts from control cells did not reproduce the complex (lane 5). We have also ascertained that the 1,25-(OH)₂D₃ receptor is not involved in either of the two complexes. This was determined by the use of monoclonal antibodies against the 1,25-(OH)₂D₃ receptor (antibody 9A7, Affinity BioReagents, Neshanic Station, N.J.). Addition of this antibody either before or after the formation of the complex did not affect its mobility or abundance.

Posttranscriptional Regulation of ck-b Expression during Differentiation of ROS 17/2.8 Cells—Data from the studies on ck-b transcription rate, mRNA abundance, and protein activity following differentiation indicate a discrepancy in the degree of enhancement among these indices. The increase in mRNA abundance far exceeds that of transcription rate, implicating the effect of increased message stability. In addition, the effect of translational modulation is implicated by the increase in mRNA abundance preceding and exceeding that in protein activity. An increase in mRNA abundance is already detectable at 6 h of incubation while that of protein is significant only at 24 h. While both peak at 48 h, the increase in mRNA abundance exceeds 5-fold while the increase in protein abundance is 2.1-
fold. Data from RNA mobility-shift assay indicate that a cytosolic trans-acting factor binds specifically to the 3'-untranslated region of the ck-b mRNA (Fig. 5). This occurs in both control and 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated cells. This interaction has previously been shown to be associated with translational modulation of the ck-b message by preventing completion of translation (19). Formation of such complexes in the 3'-untranslated region of other mRNAs is also associated with increased message stability and translational modulatory events (28).

FIG. 3. Effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3}-induced differentiation on ck-b transcription rate. Transcription rate of ck-b was analyzed by nuclear run-on studies with nuclei from control cells and cells treated with 1,25-(OH)\textsubscript{2}D\textsubscript{3}. The data shown are for cells treated for 48 h with 10 nM 1,25-(OH)\textsubscript{2}D\textsubscript{3} when ck-b mRNA abundance has peaked and reached steady state. A, autoradiographic signals for ck-b and \(\alpha\)-actin for control cells (CON) and cells treated with 10 nM 1,25-(OH)\textsubscript{2}D\textsubscript{3} for 48 hours (\(D_3\)). B, results of analysis of the signals with a laser densitometer. The top panel refers to control cells while the lower panel pertains to 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated cells. The areas under peaks 1 and 2 correlate to the intensities of the signals for \(\alpha\)-actin and ck-b, respectively. The intensity of the ck-b signal following 1,25-(OH)\textsubscript{2}D\textsubscript{3} treatment, after normalization to that of \(\alpha\)-actin, is 2.03 times that of control cells.

FIG. 4. Complexes on ck-b gene promoter. DNA mobility-shift assays on ck-b promoter region (a 421-bp fragment that spans -192 to +228 nt, relative to the cap site) with nuclear extracts from 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated and control cells. Lanes 1, 2, and 3 contain nuclear extracts from ROS 17/2.8 cells treated with 10 nM 1,25-(OH)\textsubscript{2}D\textsubscript{3} for 48 h. Lanes 4 and 5 contain extracts from control cells. Lane 1 shows that two (an upper and a lower) nuclear factor-DNA complexes are formed with nuclear extracts from 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated cells. Both complexes are competed out when >100-fold excess of unlabeled DNA corresponding to the entire 421 bp is added in lane 2. Only the lower complex, which is related to 1,25-(OH)\textsubscript{2}D\textsubscript{3} treatment, is competed out in lane 3 with a >100-fold excess of an unlabeled 228-bp fragment that corresponds in sequence to +1 to +228 nt. Only the upper complex is present when the labeled promoter is incubated with nuclear extracts from control cells in lane 4. The addition of 1,25-(OH)\textsubscript{2}D\textsubscript{3} \textit{in vitro} to nuclear extracts from control cells did not reproduce the lower complex in lane 5. U, upper complex; L, lower complex; P, free probe.

FIG. 5. Interaction of cytosolic protein with ck-b 3'-untranslated region in ROS 17/2.8 cells. RNA mobility-shift assay of S100 cytosolic extracts from control cells and cells treated with 10 nM 1,25-(OH)\textsubscript{2}D\textsubscript{3} for 48 h. Lane a contains the labeled ck-b 3'-untranslated region probe alone while lane b contains the control probe (ck-b 5' region) with the S100 fraction from 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated cells. No protected complex is seen, and the probe is digested by the added RNase T1. Lanes c and d contain the ck-b 3'-untranslated region probe with S100 fractions from 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated and control cells, respectively. Present in each lane is a protected protein-mRNA complex that is shifted in its migration when compared to probe alone (arrow).

DISCUSSION

These studies show that ck-b expression in osteoblastic cells is specifically enhanced during 1,25-(OH)\textsubscript{2}D\textsubscript{3}-modulated differentiation. Enhancement of expression is time-dependent; it is significant only after 24 h of incubation with 10 nM 1,25-(OH)\textsubscript{2}D\textsubscript{3} peaks at 48 h with a 2.1-fold increase over control, and remains elevated at the peak level for up to 96 h.

The 2-fold increase in ck-b activity reflects the overall increase in the whole cell. There is convincing evidence in the literature that ck-b expression is not uniform throughout the cell but is concentrated at subcellular structures where energy is actively expanded (5, 29–31). This includes the cytoskeleton and membrane channels, two structures in ROS 17/2.8 cells which show enhanced activity following 1,25-(OH)\textsubscript{2}D\textsubscript{3} treatment. Changes in cytoskeleton in the mature osteoblastic cell
facilitate adherence to the bone surface while increased activity of membrane pumps is related to the role of the mature osteoblast in the formation of extracellular matrix. Therefore, it is highly probable that the increase in ck-b expression following 1,25-dihydroxyvitamin D$_3$ is focused on these subcellular compartments. As such, the increase locally will be larger than the 2-fold overall increase detected in the cell as a whole.

Increased abundance of ck-b mRNA precedes and exceeds the increase in protein activity. Transcript abundance is significantly increased at 6 h and peaks at 48 h with a 5.7-fold increase over control. This degree of increase is maintained for as long as 1,25-(OH)$_2$D$_3$ incubation continues. The transcription rate of ck-b, however, is increased only 2-fold at 48 h. Hence, increased transcription can only partially account for the higher mRNA abundance; increased mRNA stability is also implicated. In addition, as the increase in protein is less than that in mRNA abundance, translational modulation is also involved.

At the transcription level, basal ck-b expression in ROS 17/2.8 cells is associated with a protein-DNA complex that involves a cis-acting element within -192 to +1 nt of the ck-b promoter. Enhancement of transcription by 1,25-(OH)$_2$D$_3$ is coupled with the formation of a second DNA-protein complex that forms on a different cis-acting element within +1 to +228 nt. This latter complex does not involve nuclear-translocated ligand-bound 1,25-(OH)$_2$D$_3$ receptor (VDR) as no recognizable VDR response element is present on the ck-b promoter, and the addition of antibodies against VDR did not cause any change in the DNA mobility-shift assay. Consequently, the effect of 1,25-(OH)$_2$D$_3$ on ck-b expression is most likely indirect, being the outcome of its effects on osteoblast differentiation. 1,25-(OH)$_2$D$_3$ is known to modulate the expression of several genes, one or more of which may then modulate ck-b expression. This interpretation is consistent with the need for an extended interval of 1,25-(OH)$_2$D$_3$ treatment before changes in ck-b expression occur. The ck-b gene promoter is known to be complex. There are multiple potential promoter sequences, and at least two different RNA polymerase complexes can form on the promoter (14, 17). Several TA-rich protein-binding regulatory regions have been described for the ck-b promoter (18). Schimmel and colleagues have shown that the DNA sequence in the region upstream of the 5'-end of the mRNA is suggestive of two superimposed promoters that contain additional sequence elements known to regulate expression of other eukaryotic genes (32). The 5'-region also has a remarkable homology to the overlapping promoters of the adenovirus E1A gene. By means of transient expression of the adenovirus E1a protein, they have been able to obtain enhanced expression of ck-b in an in vitro system (33). Hence, 1,25-(OH)$_2$D$_3$-induced differentiation could be associated with the expression of a factor (or factors) that enables complex formation on one of these enhancing elements, resulting in increased transcription. Studies are in progress to define the components of the two transcription complexes that form on the ck-b promoter following differentiation of ROS 17/2.8 cells.

In addition to transcriptional events, posttranscriptional regulation is also a factor in this system. There are several pieces of evidence that attest to the importance of posttranscriptional regulation in ck-b expression. Strauss and colleagues have shown that the 3'-untranslated region of the ck-b mRNA is >70% conserved from chicken to man (20). This extraordinary level of conservation implies a functional role for a region of the mRNA that is generally associated with posttranscriptional regulation (34). We have described a system in the U927 monoclonal cell line that involves the 3'-untranslated region in the mediation of translational regulation of ck-b expression (19). Moreover, the involvement of multiple levels of regulation would be in keeping with the need for stringent regulation of expression to ensure appropriate subcellular distribution of energy. Mature osteoblasts require changes in cytoskeletal architecture in order to adhere to periosteal and endosteal bone surfaces and limit the exposure of underlying bone to osteoclasts. Creatine kinase activity has been shown to be closely associated with the cytoskeleton where the provision of ATP is needed for microtubular movement. Cytoskeletal changes and alterations in cell shape occur in osteoblasts following incubation with 1,25-(OH)$_2$D$_3$ (6). While transcriptional events effect an overall increase in ck-b mRNA level, posttranscriptional and translational mechanisms could be involved in targeting ck-b expression to appropriate subcellular compartments with increased energy needs following differentiation.

The data that implicate involvement of posttranscriptional regulatory mechanisms are the differences in the extent of enhancement of ck-b transcription, mRNA level, and protein abundance. ck-b mRNA and protein levels peak and reach steady state at 48 h of incubation with 1,25-(OH)$_2$D$_3$. At this point, the increase over untreated cells is 2.0-, 5.7-, and 2.1-fold for transcription rate, mRNA abundance and protein activity, respectively. The discrepancy between the increase in transcription rate and mRNA abundance indicates that mRNA stability is increased. In addition, as protein abundance is increased only 2.1-fold, not all of the increased mRNA is expressed as protein. Based on these data and our earlier finding that translational repression is an important factor in ck-b regulation (19), we hypothesize that a factor binds to the 3'-untranslated region of the ck-b mRNA effecting both increased message stability and translational repression. Such a complex would explain the observation that mRNA abundance is increased to a greater extent than the increase in transcription rate and protein abundance. Supporting this hypothesis are the results of RNA mobility-shift analysis which show that a cytosolic factor in control and 1,25-(OH)$_2$D$_3$-treated ROS 17/2.8 cells interacts specifically with the 3'-untranslated region of ck-b mRNA to produce a shifted band. No such shifted band is seen with a control RNA fragment that is similar in size but unrelated in sequence (derived from the 5'-region of ck-b mRNA). This interaction could also serve as a means for targeting the expression of ck-b to subcellular compartments. It is well established that ck-b activity is compartmentalized to cellular structures where energy is being actively expanded. In addition to the cytoskeleton (5), membrane pumps are other cellular structures with localization of ck-b activity (30). This is particularly relevant during development of osteoblasts as the differentiated cell has heightened activity of its membrane pumps to facilitate the deposition of extracellular matrix and mineralization. The mechanism by which this localization of expression is mediated remains unknown. Unlike other localized proteins, posttranslational processing is not a feature of ck-b expression. The suppression of ck-b mRNA translation, mediated by the interaction of its 3'-untranslated region with a trans-acting cytosolic protein, is the probable means by which diffusion- and indiscriminate expression is prevented. Completion of translation of the mRNA is prevented by this protein-mRNA complex at the 3'-end (19). In the micromilieu of specific subcellular compartments, local factors, such as pH and ADP/ATP ratio, cause dissociation of the complex and completion of translation, resulting in increased local expression. Hentze and colleagues (35) have described a system in which cytosolic mRNA-binding proteins, present in excess, mediate translational regulation by changes in their affinity for the cis-acting element according to the presence or absence of local factors. Similarly, in Xenopus oocyte, a protein-RNA complex is formed on the 3'-untranslated region of the vegetal mRNA which targets
Acknowledgments—We thank Drs. Roland Stein and William W. Chin for helpful discussions regarding this study.

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