Analysis of the human liver/bone/kidney alkaline phosphatase promoter *in vivo* and *in vitro*

Megerditch Kiledjian and Tom Kadesch
Department of Human Genetics and The Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6072, USA

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ABSTRACT

We have carried out an analysis of the promoter for the human liver/bone/kidney alkaline phosphatase (LBK AP) gene. Using transient transfection assays, the intact promoter directs equal expression of a linked cat gene in Saos-2 cells (osteoblast-derived cells which express very high levels of endogenous LBK AP mRNA) and in HeLa and HepG2 cells (which express low levels of endogenous message). The activity of the transfected promoter apparently mimics the true *in vivo* situation since nuclear run-on assays employing Saos-2 and HeLa cells indicate that the endogenous gene is transcribed at approximately the same rate in these two cell types. Transfections of a series of 5′ deletion mutants indicate that promoter activity is dependent on multiple motifs, which possibly include several putative Sp1 binding sites and a TATA box. The LBK AP promoter also directs accurate transcription initiation in HeLa whole cell extracts and *in vitro* activities of the 5′ deletion mutants also suggest that the promoter utilizes multiple motifs.

INTRODUCTION

Alkaline phosphatases (AP) are phosphatidylinositol-linked membrane glycoproteins capable of hydrolyzing mono-phosphate esters at alkaline pH (reviewed in 1 and 2). These enzymes are widely distributed in nature ranging from prokaryotes to higher eukaryotes. In humans, there are at least three distinct AP isozymes. Two of them, placental AP and intestinal AP, are tissue specific and expressed in tissue for which they are named (2). In contrast, the third isozyme, liver/bone/kidney (LBK) AP, is more promiscuous and is present in most cell types. Although most cells contain detectable levels of LBK AP activity, both the mRNA and protein of this gene are more abundant in osteoblast derived cells (3). The molecular basis of this differential expression of the LBK gene is not known. We reasoned that the LBK AP gene may serve as an important paradigm for gene regulation since it may represent a housekeeping gene (indicated by its broad tissue distribution) that is also regulated in a cell-type specific manner (high levels of expression in osteoblasts).

The sequence of the LBK promoter (3) suggests that it is a member of the housekeeping family of promoters. Housekeeping promoters are characteristically rich in G/C content with multiple binding sites for the transcription factor Sp1 and are generally associated with genes expressed in tissues of diverse origin (4). The LBK AP gene promoter has been localized to sequences within 610 nucleotides 5′ of the major transcription start site (3). The DNA sequence reveals a potential TATA box (5) centered 30 base pairs 5′ of the start site. In addition, four consensus binding sites for the transcription factor Sp1 (6, reviewed in 7) are present. These putative Sp1 binding sites are centered 70, 82, 122 and 239 bases 5′ of the major transcription start site. Although numerous direct repeats are also present within the 610 base pair promoter fragment (suggesting that they may be functionally important), there is no obvious sequence identity to other known transcription factor binding sites (8, 9).

As a first step toward understanding regulation of the LBK alkaline phosphatase gene, we have characterized the LBK promoter *in vivo* and *in vitro* to map its functional components and to determine whether it alone is sufficient to establish the gene's differential expression.

MATERIALS AND METHODS

Plasmid DNAs

All plasmids were constructed using standard techniques (10). The plasmid designated here as pLΔ610cat is the same as pSVALBKcat previously described (3). It carries a fragment of the human alkaline phosphatase gene promoter that extends 610 base pairs 5′ (−610) and 71 base pairs 3′ (+71) of the major transcription start site (+1). The 3′ end of this DNA fragment falls within the first exon of the gene 5′ to the first ATG. The various 5′ promoter deletions were generated from pLΔ610cat by utilizing preexisting restriction sites or Bal 31 exonuclease. All of the 5′ promoter deletions retain the transcription initiation site and extend to a synthetic Hind III linker (New England Biolabs) at position +71 (3). A synthetic Bam HI linker (New England Biolabs) was inserted at a variety of restriction sites to construct deletion end points at −446 (Hinc II), −331 (BssH II), −147 (Bgl I), −60 (Sma I) and −44 (Apa I). Following digestion and linker addition, the LBK AP promoter-containing fragments were excised as Bam H1 to Hind III fragments and inserted in place of the beta globin promoter (Bam H1 to Hind III) in the plasmid pSVAβGcat(X) (11). The following plasmids were generated, respectively: pLΔ446cat(X), pLΔ331cat(X), pLΔ147cat(X), pLΔ60cat(X), and pLΔ44cat(X). Deletion endpoints for plasmids pLΔ120cat(X) and pLΔ108cat(X) were generated using Bal 31 exonuclease and contain a synthetic Bgl

II linker (New England Biolabs) at positions −120 and −108, respectively. These two promoter containing fragments were inserted into pSVAβGcat(X) as Bgl II to Hind III fragments. For plasmids that carry the SV40 enhancer ('LR' fragment of Kadesch and Berg [12]), it was inserted at the Bam HI site 3′ of the cat gene. The plasmid pSV232Acat was constructed by replacing the neo gene in pSV232Aneo (12) with the cat gene. The plasmid pGem3LD147cat was constructed by inserting the Bam HI to Eco RI fragment from pLΔ147cat(X) into the polylinker of pGem3 (Promega). This fragment contains LBK AP promoter sequences from −147 to +71 juxtaposed to cat sequences from +1 to +251. The plasmid pGem4-SV has the intact SV40 early promoter (Bam HI to Hind III, 12) inserted into the polylinker of pGem4 (Promega). The plasmid pLax, containing the adenovirus major late promoter, has been described previously (13).

Cell lines and transfections

Saos-2 cells (derived from a human osteosarcoma [14]), HeLa cells (derived from a human cervical carcinoma), and HepG2 cells (derived from a human hepatoblastoma [15]) were grown in DMEM supplemented with 10% fetal bovine serum (DMEM with low glucose for Saos-2 cells). Transfections were carried out using calcium phosphate co-precipitation essentially as described by Graham and van der Eb (16). Cells were transfected with 10 μg of the various LBK AP promoter-containing plasmids and were harvested after two days and assayed for chloramphenicol acetyltransferase (CATase; 17). In addition, five μg of a plasmid containing a β-galactosidase reporter gene (pCH110, 18) was also transfected to normalize transfection efficiencies.

Nuclear run-on assays

Isolation of nuclei, nuclear run on transcription and purification of labeled nuclear RNA were carried out essentially as described by Weber et al. (19) with the following modifications. Isolated nuclei were suspended at a concentration of approximately 6×10^7 nuclei per 200 μl of glycerol buffer (50 mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% v/v glycerol) and stored at −80 °C. Transcription reactions were carried out using 6×10^7 nuclei in 400 μl of transcription buffer (30 mM Tris-Cl pH 8, 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 2.5 mM DTT, 250 μCi [α-^{32}P]UTP [800 Ci/mmol; Amersham], 0.5 mM each ATP, GTP and CTP, 20 % v/v glycerol). Purified nuclear RNA was fragmented by adding an equal volume of 0.2 M NaOH for 30 min at 0°C, neutralized by adding a one-fifth volume of 1 M acetic acid, and then precipitated with two volumes of ethanol. Plasmid DNAs were linearized, denatured, and spotted onto nitrocellulose membranes and dried at 80°C under vacuum. Membranes were prehybridized in 50 mM Tris-Cl pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1 percent sodium dodecyl sulfate (SDS) at 42°C for one to two hours and then hybridized in 50 percent formamide, 5× Denhardt's, 5× SSPE, 0.001 percent SDS, 100 μg/ml each tRNA and poly rA, and denatured labeled nuclear RNA for 48 hours at 42 °C. Filters were washed twice in 2× SSC, 0.1 percent SDS for 5 min at room temperature and washed again in 1× SSC, 0.1 percent SDS at 60°C for 2 hours, treated with RNase A (20 μg/ml) in 1× SSC for 40 min and rinsed in 2× SSC, 0.1 percent SDS and exposed to X-ray film.

RNase protection assays

Total cellular RNA was isolated by centrifuging guanidine thiocyanate treated cell extracts through CsCl as described by Chirgwin et.al. (20). Riboprobe was generated by transcribing Bam HI-linearized pGem3LΔ147cat and pGem4-SV with T7 RNA polymerase, and RNase protection assays were carried out as described by Melton, et. al. (21) using 20 μg of total cellular RNA and 10^5 cpm of riboprobe per reaction.

In Vitro transcription assays

The preparation of HeLa whole cell extracts and *in vitro* transcription reactions were carried out essentially as described by Manley, et al. (22), except the extract was neutralized with 0.075ml of 1 M NaOH per 10g of added $(NH_4)_2SO_4$ instead of 0.1 ml. Transcription reactions were carried out in a volume of 0.02 ml at 30°C for 30 min using 84 μg extract, 300 ng LBK AP plasmid linearized with Eco R1, and 200 ng pLax linearized with Bam H1.

RESULTS

Analysis of the LBK AP promoter in vivo

To determine directly whether the LBK promoter is cell type specific, we linked it to the bacterial chloramphenicol acetyltransferase (cat) gene (17) and tested its relative expression in transient transfections of the human osteosarcoma cell line, Saos-2 (14), and Hela cells. Saos-2 cells were chosen as a representative of osteoblast cells since they retain many characteristic osteoblastic properties (23), including the expression of very high levels of LBK AP mRNA and protein. The human cervical carcinoma cell line, HeLa, was chosen as a prototype of non-osteoblast cells because those cells express several hundred fold less LBK AP mRNA and protein (M.K. and T.K., unpublished observations). Cells were transfected with plasmids carrying the LBK AP promoter and levels of cat expression were

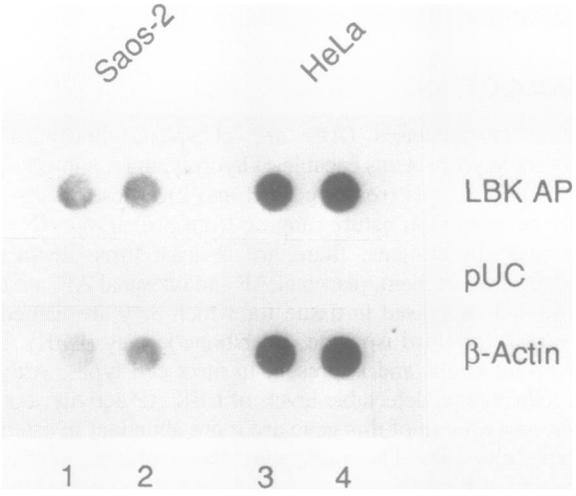

Figure 1. Nuclear run-on assays of LBK AP transcription in Saos-2 and HeLa cells. Assays were carried out as described in Materials and Methods. A total of 4×10^6 cpm of incorporated RNA from Saos-2 cells (columns 1 and 2) and HeLa cells (columns 3 and 4) was used to probe a nitrocellulose filter containing the immobilized DNAs indicated: LBK AP cDNA, pUC DNA, and genomic β-Actin DNAs (columns 1 and 3: 10 μg DNA; columns 2 and 4: 20 μg DNA). Exposure of β-Actin-containing samples to film was one-thirtieth that of the LBK AP and pUC samples.

compared to those obtained in parallel transfections using plasmids with the cat gene under control of an enhancer-deleted SV40 early promoter (pSV232Acat). We chose an enhancer-deleted SV40 early promoter for comparison because we felt it was safe to assume that this promoter would not be differentially expressed in the two cell types (it relies purely on Sp1 and TATA elements for activity). The relative levels of cat expression potentiated by the LBK promoter extending to −610, compared to the SV40 early promoter, were found not to be substantially different in Saos-2 and HeLa cells (data not shown). The LBK promoter was four to ten times more active than the enhancerless SV40 early promoter in both cell types. A LBK AP promoter fragment extending to −4500 did not appreciably increase transcription (relative to the −610 promoter) in either cell type (data not shown).

Our results indicating that the LBK AP promoter was equally active in Saos-2 and Hela cells was unexpected considering the dramatic difference in LBK AP mRNA levels in these two cell types. To more directly address this issue, and to confirm that we were not overlooking the presence of additional control elements not present in our plasmid constructs, we carried out nuclear run-on assays using Saos-2 and HeLa cells. These assays reflect the relative levels of RNA polymerase associated with a gene and, hence, its relative rate of transcription. As shown in Figure 1, using β-Actin as a control, the LBK AP gene appears to be transcribed at equal rates in Saos-2 cells and HeLa cells.

To map the DNA sequences required for promoter activity *in vivo*, and to potentially delineate any differences between Saos-2 and Hela cells, we analyzed a series of 5′ deletion mutants of the LBK AP promoter. Figure 2 illustrates the mutants that were generated and gives the results obtained from transient transfection assays. In addition to Saos-2 and HeLa cells, we transfected HepG2 cells, which also produce very low levels of LBK AP message and protein (M.K. and T.K., unpublished observations). For each cell type, sequential removal of 5′ promoter sequences resulted in a progressive decrease in transcription. The results indicate that multiple elements, in addition to the Sp1 binding sites, are necessary for full promoter activity, and that a single protein binding site does not dictate the majority of promoter activity. With the exception of what we consider to be minor cell to cell differences (i.e the differences did not correlate with the cells' relative levels of endogenous LBK AP expression), the decreasing trend in promoter activity was similar in the three cell types, supporting the idea that their transcription machineries recognize the same LBK AP promoter elements.

To confirm that the cat activity obtained with the various promoter deletions resulted from faithful transcription, we mapped the transcription start sites. HeLa cells were transfected with cat plasmids carrying the various promoter deletions in addition to the SV40 enhancer, and total cellular RNA was analyzed by RNase protection. The SV40 enhancer was inserted downstream of the cat transcription unit to give higher levels of cat mRNA, and in separate experiments (not shown) the SV40

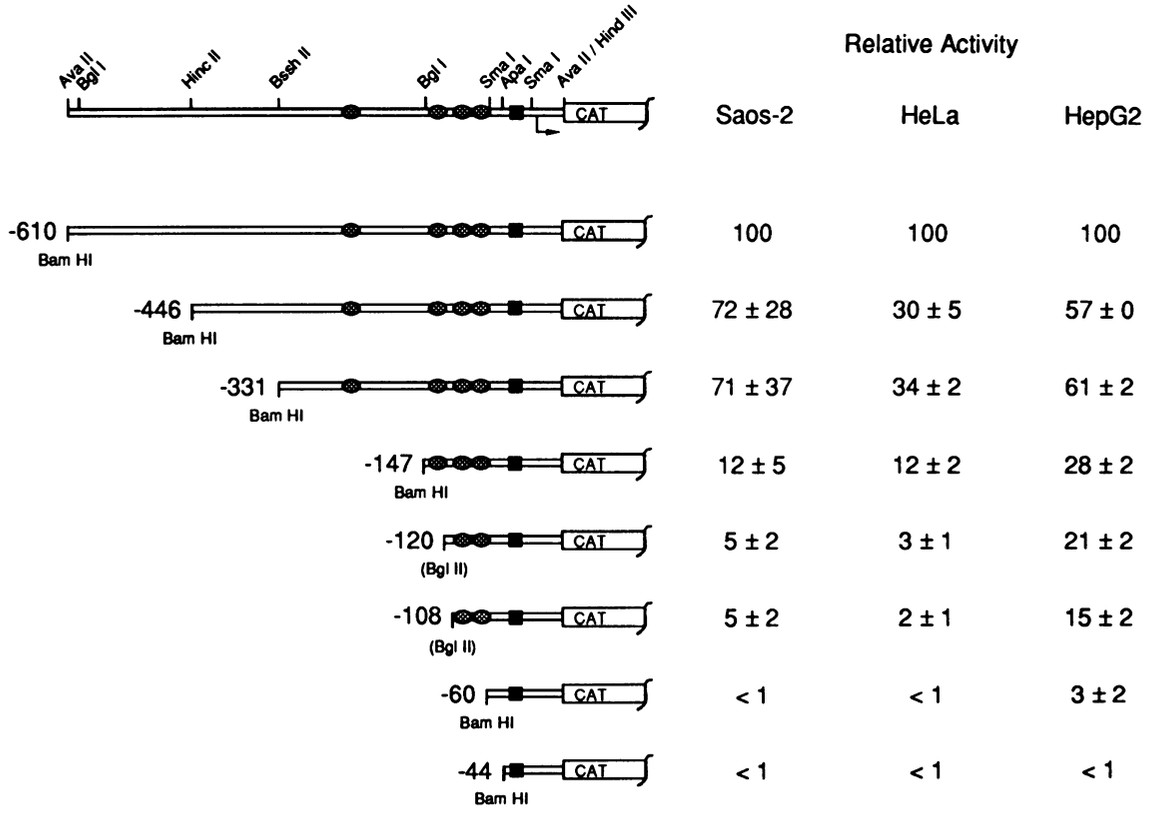

Figure 2. In vivo analysis of 5′ deletion mutants of the LBK AP promoter. Plasmids are illustrated schematically on the left with the 5′ endpoints of the LBK AP promoters indicated. Putative Sp1 recognition sequences are represented by ovals. The TATA box is shown as a filled box. Transfections of the indicated cell types and CATase assays were carried out as described in Materials and Methods. The data represent averages of five, three, and two normalized transfections for Saos-2, HepG2 and HeLa cells, respectively.

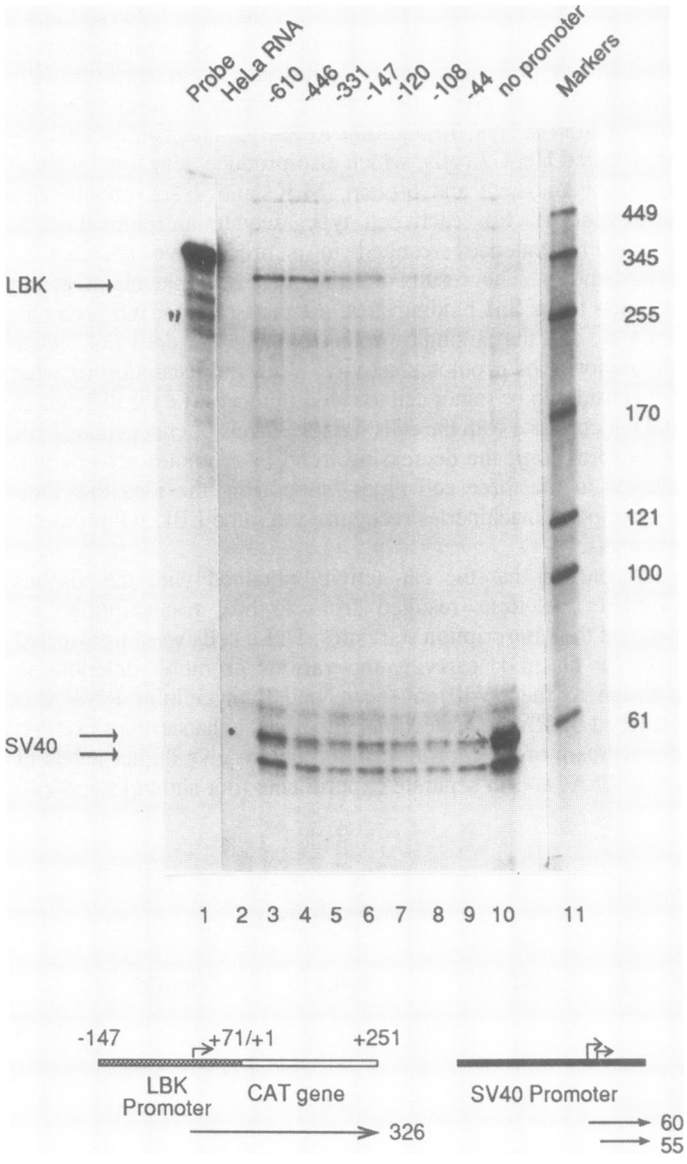

Figure 3. Analysis of transcription start sites of promoter deletions. The 5′ ends of the RNAs produced by the various promoters in HeLa cells were determined by RNase protection using 20 μg total cellular RNA. A schematic representation of the two probes used and the expected protected fragments is shown at the lower part of the figure. Correctly initiated transcripts should give rise to a protected RNA of 326 nucleotides. The doublet at the bottom of the gel, labeled 'SV40', is derived from the rabbit beta globin gene transcribed by the SV40 promoter and serves as an internal control. Lane 10 contains RNA from HeLa cells transfected with the plasmid pSV0Acat which carries no promoter elements.

enhancer did not significantly change the relative effects of the 5′ deletions between any of the cell types. As shown in Figure 3, all of the mutant promoters gave rise to correctly initiated transcripts. The 326 nucleotide protected fragment corresponds to the correctly initiated transcription start site and is only observed when a promoter is present (compare lanes 3–9 with lane 10). The doublet corresponding to the 60 and 55 nucleotide fragments is the internal control derived from a co-transfected rabbit beta-globin gene under control of the SV40 early promoter, pSβ-IVS2 (24).

Analysis of the LBK AP promoter in vitro

We also characterized the ability of the LBK AP promoter to direct transcription *in vitro*. Whole cell extracts were prepared from HeLa cells (22) and the plasmids carrying the various LBK AP promoters were tested in run off assays after they were treated with Eco RI, which cleaves 251 nucleotides into the CAT gene. A linearized plasmid carrying the adenovirus major late promoter (pLax, 13) was included in all reactions to serve as an internal control. As shown in Figure 4, the LBK AP promoter did indeed function *in vitro* as indicated by the use of the correct transcription initiation site. It was confirmed that the wild type LBK AP promoter was transcribed by Pol II due to its sensitivity to α-amanitin (compare lanes 2 and 3). Apart from a slight increase observed with the deletion end point at −331, the relative *in vitro* activities of the various 5′ deletions paralleled those obtained *in vivo*. In both cases, progressive removal of 5′ sequences resulted in a gradual decrease in promoter activity, once again indicating the presence of multiple promoter elements.

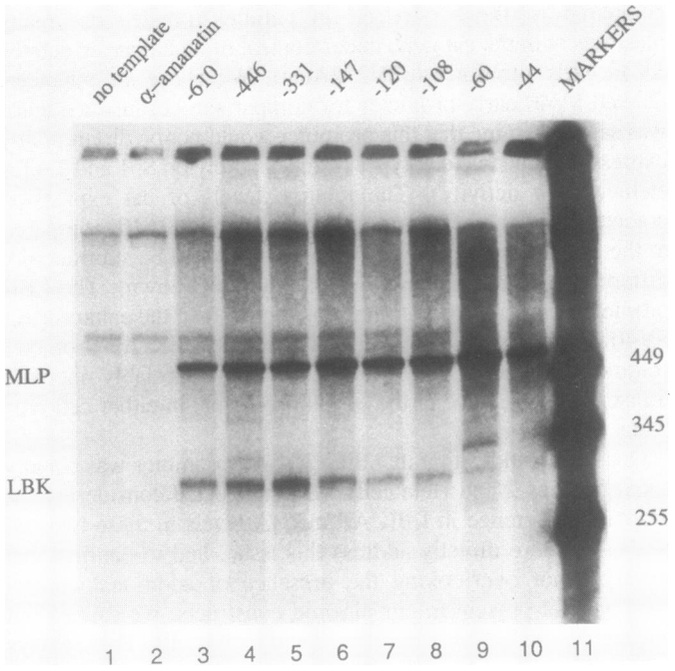

Figure 4. Analysis of the LBK AP promoter in vitro. In vitro transcription reactions using the indicated LBK AP deletions were carried out as described in Materials and Methods. The reaction of lane 2 also contained α-amanitin (1 μg per ml).

DISCUSSION

We report here the characterization of the human LBK AP promoter. Results employing transient transfections indicated that the promoter is equally active in Saos-2 and HeLa cells even though these cells express vastly different levels of LBK AP mRNA (derived from the endogenous gene). Nuclear run-on assays confirm that the endogenous gene is transcribed at roughly equal rates in the two cell types. Hence, it appears that our results with transfected promoters mimics the true *in vivo* situation.

Results from both *in vivo* and *in vitro* assays demonstrate that DNA sequences essential for promoter activity are localized within 610 nucleotides 5′ to the major transcription start site. Sequences corresponding to a putative TATA box and potential Sp1 binding sites were the only ones found to clearly correspond to known protein binding sites. Our data are consistent with the idea that the TATA and Sp1 sequences are important for promoter function *in vivo* and *in vitro*. In support of a possible role for Sp1, DNase protection experiments confirm that the proximal

binding sites are occupied *in vitro*. It is also clear that other elements are also important for promoter activity. However, due to an apparent multiplicity of sites, and perhaps low binding affinities, we have thus far been unable to clearly define these elements using either DNase protection or mobility shift assays.

Our data demonstrate that the LBK AP promoter is equally active in Saos-2 cells, HeLa cells, and HepG2 cells. Moreover, various deletions of the promoter have similar effects in all three cell types. Initially, these results were somewhat unexpected because the level of LBK AP message in Saos-2 cells is more than two orders of magnitude higher than in either HeLa cells or HepG2 cells. Nuclear run-on assays support the conclusion that the endogenous gene is transcribed at equal rates in Saos-2 and HeLa cells (and in HepG2 cells; M.K. and T.K., unpublished observations). In light of these observations, it appears as though the basal level of LBK expression is established by an inherent housekeeping promoter equally active in different cells, while differential gene expression (high expression in osteoblasts) may be mediated by a post-transcriptional mechanism.

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