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Vanadate Inhibits Expression of the Gene for Phosphoenolpyruvate Carboxykinase (GTP) in Rat Hepatoma Cells*

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Vanadate, at concentrations between 0.5 and 2 mM, rapidly decreased the basal level of P-enolpyruvate carboxykinase (GTP) (EC 4.1.1.32) mRNA and blocked the dibutyryl cyclic AMP (Bt_2cAMP)-induced increase in enzyme mRNA in both FTO-2B and H4IIE rat hepatoma cells. The concentration of vanadate necessary to inhibit the expression of this gene was similar to that required for the vanadate-mediated activation of the insulin receptor tyrosine kinase. To determine whether vanadate could inhibit PEPCK gene transcription, a series of chimeric genes containing several deletions in the P-enolpyruvate carboxykinase promoter between −550 and −68 was linked to the structural genes for either amino-3-glycosyl phosphotransferase (*neo*) or chloramphenicol acetyltransferase and introduced into hepatoma cells using three methods: (*a*) infection with a Moloney murine leukemia virus-based retrovirus, (*b*) transfection and stable selection for *neo* expression, or (*c*) transient expression of chloramphenicol acetyltransferase. In FTO-2B hepatoma cells infected with retrovirus, vanadate rapidly (within 1 h) inhibited transcription of the PEPCK-*neo* gene and blocked induction of gene expression caused by the addition of either Bt_2cAMP or dexamethasone to the cells. Vanadate was not a general transcription inhibitor since, it like insulin, stimulated the expression of the c-*fos* gene. Also, the inhibitory effect of vanadate was rapidly reversible in FTO-2B cells since PEPCK gene expression could be stimulated by Bt_2cAMP and dexamethasone after removal of vanadate. A series of 5′ deletions in the P-enolpyruvate carboxykinase promoter (−550 to +73) was ligated to the structural gene for *neo* and stably transfected into hepatoma cells. Sequences responsive to vanadate were detected between −109 and −68. This result was confirmed using H4IIE hepatoma cells transiently expressing the PEPCK-CAT gene. The most likely target for vanadate in that region of the P-enolpyruvate carboxykinase promoter is cAMP regulatory element 1 which maps from −91 to −84. A comparison of the inhibitory effects of insulin and vanadate in this system indicated a major difference in the site of action of these two compounds on PEPCK gene transcription.

Vanadate has a profound effect on a broad variety of cellular processes including the stimulation of cell differentiation (1–4), alterations in gene expression (5), and the modification of carbohydrate metabolism in both the intact animal (6, 7) and in isolated cells (8–12) and tissues (13). The most likely mechanism for the effect of vanadate on these diverse processes involves the inhibition of cellular phosphotyrosine phosphatases (14, 15) and the activation of specific protein kinases (14, 15). Vanadate shares many common metabolic features with insulin. It increases the rate of 2-deoxyglucose transport into adipocytes (11, 16), induces differentiation of quiescent Swiss 3T3 cells and 3T6 cell lines (17), increases the phosphorylation of tyrosine on the insulin receptor (8, 9), and normalizes the blood glucose concentration when administered orally to diabetic animals (6, 18–20). Many of these metabolic effects of vanadate can be linked directly to the activation of the insulin receptor by an inhibition of phosphotyrosine phosphatases.

The cytosolic form of P-enolpyruvate carboxykinase is a major site of glucose homeostasis in vertebrates (21), and its activity is regulated by glucagon and insulin. The expression of the gene for this enzyme is markedly decreased by insulin (22, 23) and phorbol ester (24), an effect which is mainly due to an inhibition of PEPCK[1] gene transcription (23, 25). Regulatory elements in the PEPCK promoter, which are responsive to a number of hormones including cAMP (26–29), glucocorticoids (27–29), and insulin (23, 33), are contained in a segment of the promoter between −550 and +73. Several of these regulatory elements have been shown to bind nuclear proteins (30, 31) which may be involved in the control of PEPCK gene transcription. In the present report, we have examined the effect of vanadate on the transcription of the PEPCK gene and have identified specific sequences in the PEPCK promoter which are responsive to vanadate. We find that vanadate, while inhibiting the transcription of the gene, does so at a site on the PEPCK promoter distinct from that of insulin.

EXPERIMENTAL PROCEDURES

Materials—The random labeling kit and all restriction enzymes were obtained from Boehringer Mannheim. GeneScreen *Plus* and [^{32}P]dCTP (3000 Ci/mmol) were purchased from Du Pont-New England Nuclear. All media, sera, and G418 were from Gibco Laboratories, and sodium orthovanadate was purchased from Sigma. All other reagents used were of the highest purity available. The FTO-2B cells used in this study were a gift from Dr. Keith Fournier, Fred Hutchison Cancer Center, Seattle, WA (26, 32). The retroviral vector, pLJ, and the Ψ2 cells were from Dr. Richard C. Mulligan, Whitehead Institute,

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[1] The abbreviations used are: PEPCK, P-enolpyruvate carboxykinase; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's minimal essential medium, Bt_2cAMP, dibutyryl cyclic AMP; CRE-1, cAMP regulatory element 1; TAT, tyrosine aminotransferase; TK, thymidine kinase.

Massachusetts Institute of Technology, Boston, MA. The tyrosine aminotransferase cDNA was provided by Dr. Gunther Schutz, University of Heidelberg, Heidelberg, West Germany and the c-*fos* by Dr. Thomas Curran, Roche Institute, Rahway, NJ.

Retroviral Vectors—The retroviral vector, pLJPCK, was constructed from the parent vector pLJ by the insertion of a segment of the PEPCK promoter (−550 to +73) in place of the SV40 promoter (see *bottom* of Fig. 3 for map). A detailed discussion of the properties of pLJ and pLJPCK has been presented previously (33).

Infectious retroviruses were generated by transfection of pLJPCK (10 μg of retroviral DNA) into Ψ2 cells, a viral packaging cell line (34). Cells expressing *neo* were selected in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum and 1 mg/ml G418. The titer of the infectious retrovirus (vLJPCK) produced by the Ψ2 cells was approximately 8×10^6 colony-forming units/ml. To infect cells, 1 ml of medium containing the retrovirus was added to approximately 10^5 FTO-2B cells, together with 8 μg/ml Polybrene and incubated for 5 h at 37 °C. The cells were then grown in DMEM containing 5% fetal calf serum and 5% calf serum for 2 days prior to the addition of medium containing G418. For more details see Hatzoglou *et al.* (33).

DNA Probes—The following probes were used for Northern analysis of RNA: PEPCK, a 1.1-kilobase *Pst*I-*Pst*I fragment from the 3′ end of the PEPCK DNA (35); *neo*, 1-kilobase *Bgl*II-*Eco*RI fragment from the *neo* gene (33); tyrosine aminotransferase cDNA, 600-base pair *Pst*I-*Pst*I fragment which includes the 3′ end of the tyrosine aminotransferase cDNA (36); c-*fos* cDNA, 1.0-kilobase *Bgl*II-*Sac*I fragment of the cDNA. These DNA probes were labeled using [α-^{32}P] dCTP, following the method of random oligopriming as described by the manufacturer. The specific activity of the DNA probe labeled in this manner was approximately 10^9 cpm/μg DNA.

Isolation and Analysis of Cellular RNA—Isolation of total RNA and Northern analysis were carried out by standard procedures which have been described previously (37, 38). The levels of mRNA for actin and glyceraldehyde-3-phosphate dehydrogenase, which were not altered by vanadate treatment, were used to normalize for variations in RNA transfer during Northern blotting.

Construction of PEPCK Chimeric Genes—The construction of the serial deletions of the PEPCK promoter by digestion with Bal-31 and their subsequent ligation to *neo* has been described previously (28). To construct the PEPCK-chloramphenicol acetyltransferase vectors, the same shortened segments of the PEPCK promoter were removed from the PEPCK-*neo* vectors by digestion with *Xba*I-*Bgl*II (28) and ligated into a polylinker in front of the CAT gene. The PEPCK-CAT gene was removed by *Xba*I-*Pst*I digestion and placed in the polylinker of the PTZ18R vector. Further details of the chloramphenicol acetyltransferase vector construction will be given elsewhere.[2]

Hormonal Treatment—FTO-2B cells were maintained in serum-free medium for 18 h prior to the addition of hormones. The cells were then incubated with either 0.5 mM Bt_2cAMP plus 1 mM theophylline, 1 μM dexamethasone, or 50 nM bovine insulin. The concentration of vanadate used in individual experiments is indicated in the text.

Cell Transfection and the Determination of Chloramphenicol Acetyltransferase Activity—H4IIE hepatoma cells were transfected with plasmids containing the various PEPCK-CAT chimeric genes. Cells at 90% confluency were treated with trypsin and resuspended together with calcium phosphate-precipitated DNA to ensure equal transfection efficiency. The cells were then plated and after 5 h shocked with 10% glycerol for 2 min. Approximately 36 h after transfection, the cells were incubated with either 0.5 mM 8-bromo-cAMP plus 1 mM theophylline or 8-bromo-cAMP plus theophylline and 0.25 mM vanadate for 15 h. Cells were scraped from the plates lysed by freeze-thawing, and the cellular debris was removed by centrifugation. The determination of chloramphenicol acetyltransferase activity was performed using an equal amount of protein from each plate with 0.1 μCi of [^{14}C]chloramphenicol in each assay (39).

RESULTS

Effects of Vanadate on PEPCK Gene Expression in Hepatoma Cells—The effect of vanadate on the regulation of PEPCK gene expression was studied using FTO-2B and H4IIE hepatoma cells. It has been demonstrated that these cell lines respond to insulin, and the inhibitory effects of this hormone on transcription of the PEPCK gene have been reported previously (22, 33). Treatment of FTO-2B cells with 2 mM vanadate or with insulin for 4 h decreased the concentration of PEPCK mRNA when analyzed by Northern blotting (Fig. 1*A*). Incubation of the cells with Bt_2cAMP plus theophylline increased the level of PEPCK mRNA while vanadate blocked this induction (Fig. 1*A*). The same effect was noted after treatment of H4IIE cells with Bt_2cAMP plus vanadate (data not shown). It has been demonstrated previously that insulin can rapidly induce the transcription of c-*fos* (40). Like insulin, 2 mM vanadate increased the concentration of c-*fos* mRNA in FTO-2B cells within 30 min after treatment (Fig. 1*B*), indicating that gene expression in the hepatoma cells could respond to vanadate in both a positive and negative manner.

Vanadate blocked the inductive effects of Bt_2cAMP on PEPCK mRNA in a dose-dependent manner. When FTO-2B cells were incubated with Bt_2cAMP plus increasing concentrations of vanadate, the inhibitory effect was observed at concentrations of vanadate of 0.5 mM or greater (Fig. 2). This concentration approximates the minimal levels of vanadate which activate the tyrosine kinase of the insulin receptor (41, 42). No effect of vanadate on actin or glyceraldehyde-3-P dehydrogenase mRNA levels was observed (data not shown). Thus, vanadate exerts an insulin-like effect on the expression of the endogenous PEPCK gene in hepatoma cells.

Vanadate Inhibition of PEPCK Gene Transcription—To determine whether the effect of vanadate on PEPCK mRNA involved sequences in the 5′-flanking region of the PEPCK gene, we used FTO-2B cells infected with replication-incompetent virus containing the PEPCK promoter (−550 to +73) ligated to the structural gene for *neo*. This retroviral vector was chosen because the PEPCK promoter present in the provirus is inhibited by insulin in a manner similar to the endogenous PEPCK gene (33). Vanadate regulation of the chimeric PEPCK-*neo* gene in FTO-2B cells infected with vLJPCK was analyzed by Northern blotting. Treatment of the cells for 4 h with Bt_2cAMP plus theophylline or with dexamethasone caused a 6–7-fold induction in the level of a 3.2-kilobase *neo* RNA (Fig. 3*A*). This size mRNA is produced when transcription is initiated at the PEPCK promoter and the newly synthesized RNA is polyadenylated within the 3′ long terminal repeat. Like insulin, vanadate (2 mM) markedly decreased the basal level of the expression of the chimeric

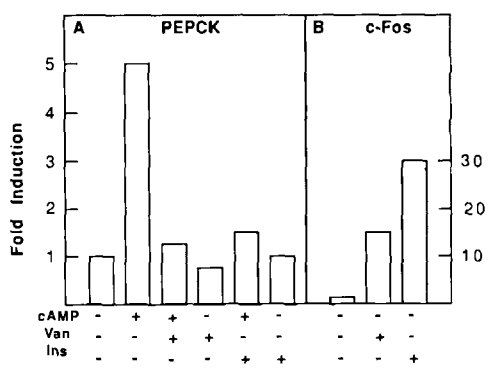

FIG. 1. **The effects of vanadate on PEPCK and c-*fos* mRNA in FTO-2B cells.** *A*, the levels of RNA for endogenous PEPCK and c-*fos* were determined by Northern analysis of RNA isolated from cells which were treated for 4 h with medium (no hormones added) (−), 2 mM vanadate (*Van*), 0.5 mM Bt_2cAMP (*cAMP*), and 1 mM theophylline or 50 nM insulin (*Ins*). The data is expressed as -fold induction over the control. *B*, the induction of c-*fos* mRNA after 30 min of treatment with 2 mM vanadate or 50 mM insulin is shown. Details on the procedures for the extraction and analysis of RNA are provided under "Experimental Procedures."

[2] J. S. Liu, manuscript in preparation.

PEPCK-*neo* gene and blocked stimulation by Bt_2cAMP or dexamethasone of transcription from the PEPCK promoter (Fig. 3*A*). As is shown in Fig. 3*A*, the expression of the endogenous PEPCK gene was also inhibited by vanadate. In this infected cell line, the basal expression of the endogenous PEPCK gene is increased as was described previously (33). This elevation resulted in a more pronounced decrease in the level of the endogenous mRNA by both vanadate and insulin (33). These results indicate that vanadate can inhibit transcription by acting at sequences in the 5′-flanking region of the PEPCK promoter.

The effects obtained after incubation of the FTO-2B cells infected with vLJPCK for 30 min, 1 h, or 2 h with 2 mM vanadate, Bt_2cAMP, or both compounds together is shown in Fig. 3*B*. The Northern blot was hybridized with *neo*, PEPCK, and tyrosine aminotransferase DNA probes. Like insulin, vanadate caused a marked inhibition in the Bt_2cAMP-stimulated expression of all three genes within 1 h.

The effect of insulin on PEPCK gene transcription is rapidly reversible following insulin removal from the medium in which the hepatoma cells are grown (43). We next determined if PEPCK gene transcription could be stimulated following vanadate removal from the cells. FTO-2B cells infected with vLJPCK were incubated for 4 h in either DMEM, with no serum or hormones added, or DMEM containing 2 mM vanadate or 50 nM insulin. Four h later, the medium was removed and the cells washed. Plain medium or medium containing 0.5 mM Bt_2cAMP or 1 μM dexamethasone was added, and the cells were incubated for an additional 4 h. RNA was isolated from the cells and the concentration of *neo*, PEPCK, and tyrosine aminotransferase mRNA determined. Pretreatment of the cells with vanadate caused a decrease in the concentration of the PEPCK and tyrosine aminotransferase mRNA, as well as PEPCK-*neo* mRNA transcribed from the chimeric PEPCK-*neo* gene in the provirus (Fig. 4*A*, *lanes 1* and *4*). Vanadate also blocked the induction of PEPCK, *neo*, and TAT gene transcription caused by Bt_2cAMP or dexamethasone (Fig. 4*A*, *lanes 2, 3* and *5, 6*). However, the extent of induction of mRNA by both Bt_2cAMP and dexamethasone was the same in control and in vanadate-pretreated cells, indicating that the effect of vanadate on the FTO-2B hepatoma cells was reversible. Pretreatment of the cells with insulin resulted in a similar pattern of response to hormones. Insulin inhibited the normal induction of *neo*, PEPCK, and TAT gene expression caused by dexamethasone or Bt_2cAMP (Fig. 4*B*, lanes 2, 3 and *5, 6*). These experiments were also performed using FTO-2B cells which were not infected with the retrovirus, and similar results were obtained for PEPCK and TAT gene expression (data not shown).

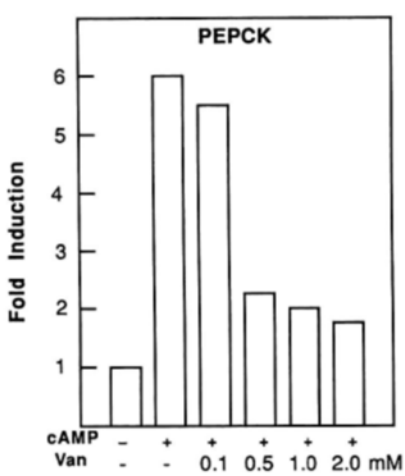

FIG. 2. **The effect of increasing concentrations of vanadate on Bt_2cAMP induction of PEPCK mRNA.** FTO-2B cells were treated with 0.5 mM Bt_2cAMP and 1 mM theophylline or with Bt_2cAMP plus the indicated concentrations of vanadate for 4 h. RNA was extracted from the cells, and the concentration of PEPCK mRNA was determined by Northern blotting using PEPCK cDNA as a hybridization probe.

Regions of the PEPCK Promoter Responsive to Vanadate— In order to compare the region(s) of the PEPCK promoter responsive to vanadate and insulin, a series of chimeric PEPCK-*neo* genes containing segments of the PEPCK promoter (from either −550, −335, or −174 to +73) was introduced into FTO-2B cells via retroviral infection. The effect of vanadate on transcription from these truncated promoter fragments was determined by measuring changes in *neo* mRNA. Vanadate (2 mM) inhibited both the basal level of PEPCK-*neo* gene transcription as well as the Bt_2cAMP or dexamethasone induction of all three fusion genes (Fig. 5), suggesting that sequences further 3′ are involved in the vanadate effect. On the other hand, insulin was unable to consistently block the induction of either the −355 or the −174 infected PEPCK-*neo* genes. In one series of infections, both of the chimeric genes were inhibited by insulin, but in two other infections insulin had no effect (data not shown). This contrasts with vanadate which consistently blocked the effect of cAMP and dexamethasone in all infected lines.

It has been previously shown that insulin did not inhibit either basal or cAMP-stimulated transcription from the PEPCK promoter of a chimeric PEPCK-TK or PEPCK-*neo* gene which had been stably introduced into hepatoma cells (27). However, we decided to use this system 1) to determine if vanadate could inhibit transcription from the PEPCK

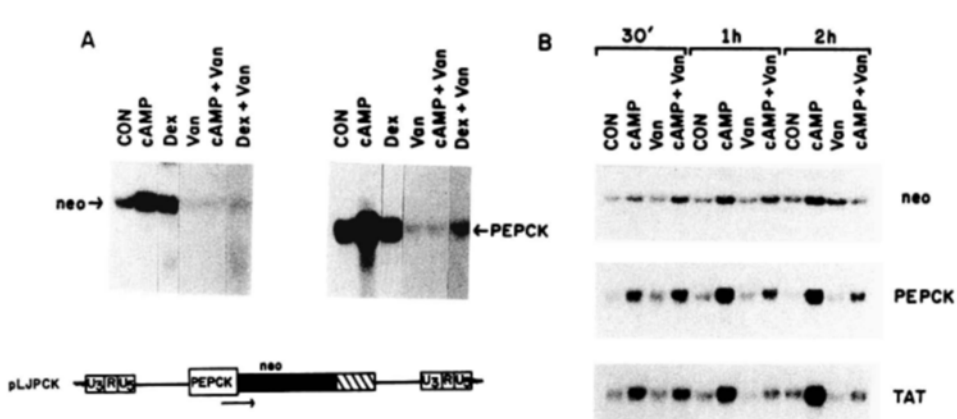

FIG. 3. **Effects of vanadate on FTO-2B cells infected with vLJPCK.** FTO-2B cells were infected with vLJPCK and maintained in culture with G418. *A*, the levels of *neo* and endogenous PEPCK RNA were determined by Northern analysis using RNA isolated for cells treated for 4 h with 2 mM vanadate and with 0.5 mM Bt_2cAMP or 1 μM dexamethasone (*Dex*), with or without 2 mM vanadate (*Van*). The vector used for the infection of the FTO-2B cells is shown at the *bottom* of the figure. *B*, the time dependence of the effect of 1 mM vanadate on the basal or 0.5 mM Bt_2cAMP-induced *neo*, endogenous PEPCK, or tyrosine aminotransferase mRNA levels.

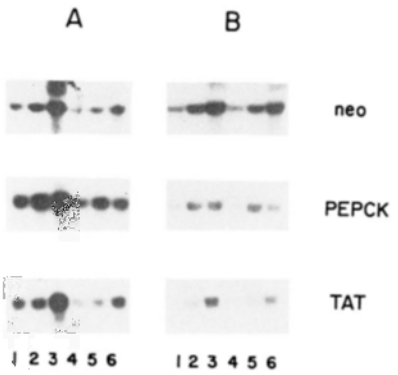

FIG. 4. **Effects of Bt_2cAMP and dexamethasone on *neo*, PEPCK, and tyrosine aminotransferase mRNA levels in retrovirally infected FTO-2B cells which had been pretreated with vanadate or insulin.** FTO-2B cells infected with vLJPCK were treated with medium (no serum or hormone added) (Fig. 4, *A* and *B, lanes 1–3*), 2 mM vanadate (Fig. 4*A, lanes 4–6*), or 50 nM insulin (Fig. 4*B, lanes 4–6*) for 4 h. The cells were then washed three times with plain medium, and then to each group of cells was added plain medium (Fig. 4, *A* and *B, lanes 1* and *4*) or medium containing 0.5 mM Bt_2cAMP (Fig. 4, *A* and *B, lanes 2* and *5*) or 1 μM dexamethasone (Fig. 4, *A* and *B, lanes 3* and *6*). After 4 h the levels of *neo*, PEPCK, and tyrosine aminotransferase mRNA were measured.

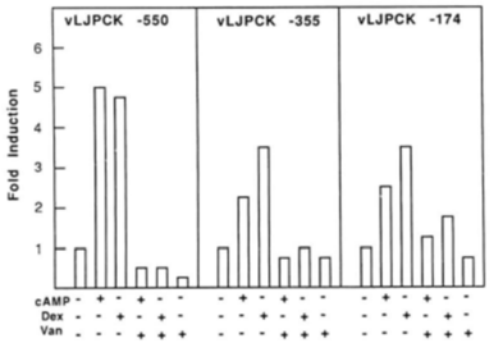

FIG. 5. **The effects of vanadate on the expression of the PEPCK-*neo* gene in FTO-2B cells infected with either vLJPCK (−550), vLJPCK (−355), or with vLJPCK (−174).** FTO-2B cells were infected with retrovirus containing segments of the PEPCK promoter (either −550, −355, or −174 to +73) linked to the *neo* structural gene. The concentration of mRNA for *neo* was determined by Northern analysis of RNA isolated from these cells after treatment for 4 h with 2 mM vanadate (*Van*) or with 0.5 mM Bt_2cAMP (*cAMP*) and 1 μM dexamethasone (*Dex*) in the presence or absence of vanadate.

promoter in cells in which the chimeric PEPCK-*neo* gene was introduced by stable transfection and 2) to identify the sequences involved in the vanadate inhibition of PEPCK transcription. A series of promoter deletions mapping at the 5′ end from −550, −355, −174, −109, and −68 to +73 at the 3′ end of the promoter (28) was linked to the *neo* structural gene and transfected into FTO-2B cells. In these experiments, transcription from the PEPCK promoter was stimulated by the addition of 0.5 mM Bt_2cAMP to the cells, and the inhibitory effect of 2 mM vanadate on the concentration of *neo* RNA was determined 4 h later (Fig. 6). Vanadate blocked Bt_2cAMP-stimulated transcription in deletions from −550 to −109 in the PEPCK promoter but had no effect on transcription from −68. This segment of the promoter has been shown previously to be transcriptionally unresponsive to cAMP (27). The absence of an effect of insulin on the stably transfected −550 PEPCK-*neo* gene is shown.

An alternative method for measuring the effect of vanadate on PEPCK gene transcription involves transient expression

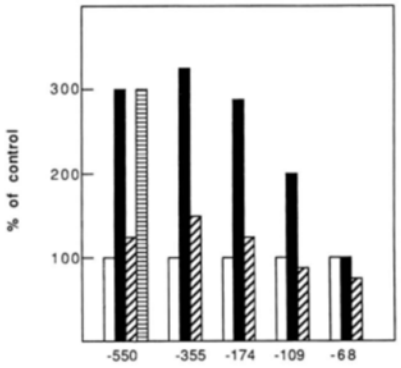

FIG. 6. **The effect of vanadate on FTO-2B cells stably transfected with plasmids containing PEPCK-*neo*.** FTO-2B cells were transfected with chimeric PEPCK-*neo* genes which contained segments of the PEPCK promoter regulatory regions (−550 to +73, −355 to +73, −174 to +73, −109 to +73, and −68 to +73) in p5′-PC*neo* (28). RNA was isolated after 4 h of treatment with serum-free medium (*open bars*) or with 0.5 mM Bt_2cAMP in the absence (*solid bars*) or presence (*striped bars*) of vanadate and analyzed by Northern blotting. The effect of insulin on the −550 PEPCK-*neo* vector is shown (*horizontal stripes*). Results are presented as a percentage of the control RNA from untreated cells.

of chimeric PEPCK-CAT genes in H4IIE hepatoma cells. Bt_2cAMP addition to the cells caused a 2-fold stimulation of chloramphenicol acetyltransferase activity with PEPCK promoter deletions from −490 to −109 but not with the promoter deleted to −68. The same relative inhibitory effect of vanadate on Bt_2cAMP-stimulated transcription of the PEPCK-CAT chimeric genes was noted in all promoter deletions through −109. Thus, two independent methods for measuring the action of vanadate on the PEPCK promoter indicated an effect on sequences between −109 and −68. The only known regulatory element in this region of the promoter is CRE-1, an element which is involved in the control of both basal and cAMP-stimulated transcription of the PEPCK gene (28).

DISCUSSION

Despite an extensive literature on the effects of vanadate on cellular metabolism and differentiation, there is little known about its effects on gene expression. Wice *et al.* (4) reported that vanadate increased the level of c-*fos* mRNA in BC3H1 cells within 30 min when the cells were induced to differentiate. In this report we demonstrate that vanadate, like insulin, can markedly decrease transcription of the PEPCK gene and can also induce c-*fos* in FTO-2B hepatoma cells. Since vanadate is considered an insulin mimetic agent, it may share with insulin a common mechanism of regulation of gene transcription.

A key step in insulin action is tyrosine autophosphorylation of the β-subunit of its receptor (44–47). It has been reported that vanadate also increases phosphorylation of the insulin receptor (8, 9), presumably either by inhibiting the dephosphorylation of phosphotyrosine residues on the receptor (48) or activating tyrosine kinases (9). Activation of the insulin receptor kinase requires vanadate concentrations of 0.5 mM or higher (9, 41, 42), a concentration similar to that required to block transcription of the PEPCK gene. Similar doses of vanadate are needed to increase the phosphate content of the 15-kDa phosphotyrosyl protein, pp15 (49), a protein that is phosphorylated on tyrosine residues in response to insulin (50). The function of this protein is unknown, but it has been suggested that it could be an intermediate of insulin-stimulated signal transmission (49). Finally, the metabolic actions of vanadate on glucose and glycogen metabolism in isolated

cells and tissues require similar concentrations of vanadate to be effective (7, 10, 16).

Effect of Vanadate on the PEPCK Promoter—In this paper we have used three different methods to test the effect of vanadate on transcription from the PEPCK promoter in hepatoma cells in order to exploit the advantages that each provides. Previous attempts to identify an insulin-specific region in the PEPCK promoter using stable transfection/selection provided misleading results. Wynshaw-Boris *et al.* (27) reported that the region of the PEPCK promoter between −550 and +73 was not responsive to the inhibitory effect of insulin on the transcription of a PEPCK-TK chimeric gene stably transfected into FTO-2B cells. Subsequently, Magnuson *et al.* (23), using a chimeric PEPCK-CAT gene introduced transiently into H4IIE cells, were able to demonstrate that insulin blocked glucocorticoid-stimulated transcription from the PEPCK promoter (−650 to +68). Likewise, Hatzoglou *et al.* (33), using the retroviral vector employed in the current study, showed that insulin blocked both the basal and cAMP-stimulated gene transcription from the same segment of the PEPCK promoter. The reason for this difference in response to insulin of the transfected PEPCK promoter is not clear, but it may be related to the cell selection required for stable transfection and/or the site of integration of the transfected gene in the host cell genome. In order to avoid similar complications in our studies with vanadate, we have used several techniques to introduce various segments of the PEPCK promoter into hepatoma cells.

All of the procedures used to determine the effect of vanadate on PEPCK gene expression in the present study indicate an acute inhibition of both basal and cAMP-stimulated gene transcription. Using PEPCK promoter deletions introduced both stably and transiently, we have mapped the vanadate-responsive region in the promoter to between −109 and −68. The only known transcription regulatory element in this area of the promoter is CRE-1 (−91 TTACGTCA −84), which has been shown by Quinn *et al.* (31) to be important for both cAMP-responsive and basal transcription from the PEPCK promoter. This core sequence is part of a coordinated response region in the PEPCK promoter which includes elements mapping at −155 to −135 (CRE-2), −231 to −200 (P3), and −320 to −269 (P4). Roesler *et al.* (30) have shown that these four regions, which share considerable sequence identity, bind the same protein(s) isolated from rat liver nuclei. While CRE-1 is sufficient to confer cAMP responsiveness on a neutral promoter in gene transfection experiments (28), the highest level of response to cAMP was noted using a segment of the promoter (−416 to −61) which contains the four CRE-like regions in the intact promoter.

If vanadate acts on CRE-1 to alter PEPCK gene transcription, it may do so by modifying nuclear proteins such as CREB (51, 52), a 43-kDa phosphoprotein which has been isolated from rat brain (51) and human placenta (52) and shown to induce transcription from the rat somatostatin promoter in an *in vitro* assay (53). Phosphorylation of CREB by the catalytic subunit of protein kinase A mediates this induction. Recently, we have found that purified C/EBP (54, 55) also binds to CRE-1, P3, and P4 with a DNase I footprinting pattern similar to that noted with a nuclear protein purified by CRE-1 affinity chromatography.[3] C/EBP is found in placenta, liver, adipose tissue, mammary gland, and small intestine (57) and is involved in the transcriptional regulation of highly tissue-specific genes such as albumin (55). In addition, CREB binds to the CRE-1 sequences but not to the more 5′ sites which bind C/EBP.[3] We are currently trying to determine which proteins are bound *in vivo* and their interrelationship in the response to cAMP. A better understanding of the possible interaction of vanadate and proteins regulating PEPCK gene transcription will require the use of a cell-free transcription system capable of responding to cellular proteins modified *in vivo* by vanadate. The acute and very marked effect of vanadate on a number of cAMP-regulated genes noted in this report could indicate that vanadate will be an important tool for dissecting the complexities involved in the phosphorylation of nuclear proteins involved in regulating gene nuclear transcription.

Do Vanadate and Insulin Share a Common Mechanism—Both vanadate and insulin inhibit the transcription of the PEPCK gene, but do they share a common mechanism? Several lines of evidence suggest different pathways. Recent studies by Forest *et al.* (58) have delineated a region of the PEPCK promoter between −450 and −277 as involved in the insulin response. Insulin did not consistently inhibit transcription of a chimeric PEPCK-*neo* gene introduced into FTO-2B cells with a retroviral vector when promoter segments of less than −355 were included. This contrasts with our findings with vanadate, which markedly inhibited both basal and cAMP-stimulated PEPCK gene transcription with promoter deletions as short as −109 (see Figs. 6 and 7). In addition, vanadate can inhibit transcription of stably transfected genes containing PEPCK-*neo*, while insulin cannot. It is possible that vanadate and insulin initiate a common signal, for example the phosphorylation of the insulin receptor or pp15, either of which could then act to inhibit transcription at different sites on the PEPCK promoter. However, vanadate undoubtedly has other effects such as increasing the formation of inositol phosphates (59) and the uptake of Ca^{2+} (60), which could also alter the transcription of the PEPCK gene, again at a site different from that of insulin. These additional effects of vanadate may account for its ability to inhibit transcription from a relatively small segment of the PEPCK promoter. However, vanadate does not merely inhibit genes which are sensitive to cAMP since the transcription of c-*fos* is stimulated by vanadate. The expression of another insulin-regulated gene, tyrosine aminotransferase, is inhibited by vanadate but stimulated by insulin (56), suggesting a different mechanism for the two compounds. Thus, while vanadate is

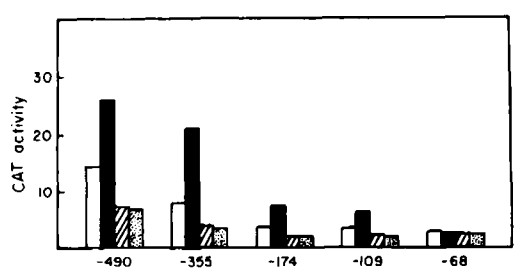

FIG. 7. **The determination of regions of the PEPCK promoter responsive to vanadate after transient transfection of PEPCK-chloramphenicol acetyltransferase into hepatoma cells.** H4IIE hepatoma cells were transfected transiently with a series of chimeric PEPCK-CAT genes which contained different segments of the PEPCK promoter regulatory region (−490 to +73, −355 to +73, −174 to +73, −109 to +73, and −68 to +73) linked to the CAT structural gene. Cells were treated for 16 h with plain medium (*open bars*), 0.5 mM 8-bromo-cAMP plus 1 mM theophylline (*solid bars*), or 8-bromo-cAMP plus 0.25 mM vanadate (*striped bars*). The cells were then harvested and chloramphenicol acetyltransferase activity measured as outlined under "Experimental Procedures." All assays were carried out in duplicate, and the experiments were repeated three times.

[3] Park, E. A., Roesler, W. J., Liu, J., Klemm, D., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W. (1990) *Mol. Cell. Biol.*, in press.

often referred to as an insulin-mimetic agent, it appears to exert a negative effect on the PEPCK promoter via a mechanism different from that of insulin.

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