Role of the promoter in the sensitivity of human thymidine kinase to lack of Zn^{2+}

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Previous studies had indicated that lack of Zn^{2+} inhibits the expression of thymidine kinase activity and produces a corresponding reduction in the concentration of its mRNA. The present investigations have shown that with human thymidine kinase this is associated with increased binding of a specific protein to the gene's promoter in the region between −55 and −83 bp 5′ to the transcription initiation site. A second binding site for the protein is present within the sixth exon of the human thymidine kinase gene.

INTRODUCTION

Thymidine kinase (EC 2.7.1.21) activity in mouse 3T3 cells decreased when the availability of Zn^{2+} was depressed by addition of a strong chelator, and the loss of activity was associated with a corresponding reduction in the cellular concentration of thymidine kinase mRNA [1]. This effect was equally apparent with TK–Syrian hamster cells which had been transfected with constructs coding for human thymidine kinase even when the human thymidine kinase promoter activating the construct was restricted to the first 83 bp 5′ to the transcription start site [2].

The present studies of modified human thymidine kinase constructs identified regions of the thymidine kinase promoter which influenced its sensitivity to Zn^{2+} availability but initially failed to reveal the underlying basis for this sensitivity. However, gel-mobility-shift analysis of protein binding within the critical region of the human thymidine kinase promoter demonstrated increased binding of a specific fraction when extracts were prepared from Zn^{2+}-deficient cells. The DNA-binding site for this complex has been identified. Furthermore, a similar site is present within the sixth exon of the human thymidine kinase gene. The previously observed effects of lack of Zn^{2+} on production of thymidine kinase activity from the plasmids containing modified thymidine kinase promoters were explicable in terms of a Zn^{2+}-dependent protein binding to these sites and inhibiting transcription of the gene.

EXPERIMENTAL

BHK TK^- cells (European Collection of Animal Cell Cultures, Porton Down, Wilts., U.K.) were cultured in Dulbecco's Eagle's medium supplemented with penicillin (50 units/ml), streptomycin (50 units/ml), fungizone (2.5 μg/ml) and 12% (v/v) foetal calf serum (Life Technologies, Paisley, Scotland, U.K.). The cells were grown in Petri dishes at 37 °C in an atmosphere containing air/CO_2 (9:1, v/v). Stably transfected cell lines were obtained by transfection of the cells with plasmids containing human thymidine kinase minigene constructs using a calcium phosphate method [3]. Stably transfected cells were selected and maintained in medium further supplemented with HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), and the final cultures were derived from a mixture of at least 100 clones thereby reducing any bias associated with differences in individual clones.

Most of the plasmids used for transfection contained a minigene construct [4] that consisted of the first two exons and introns of the human thymidine kinase gene with the coding region completed by the human thymidine kinase cDNA sequence. These minigenes were activated by one of the modified human thymidine kinase promoters indicated in Figure 1. A further group of cells was transfected with a plasmid containing the full human thymidine kinase cDNA sequence under the influence of the simian virus (SV)40 early promoter.

Thymidine kinase activity

The effects of Zn^{2+} deprivation on thymidine kinase activity were investigated with each of the transfected cell lines. Each ex-

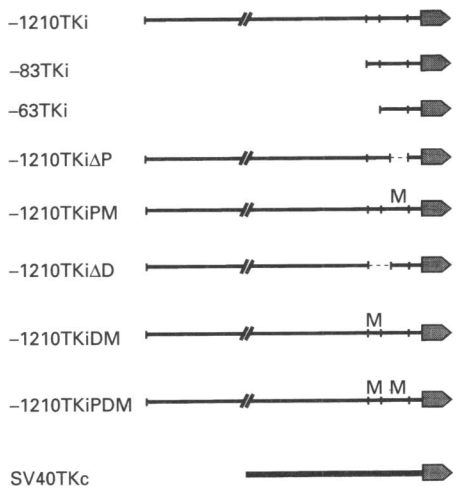

Figure 1 Promoters used to express human thymidine kinase in BHK TK^- cells

Most of the promoters were based on the −1210 bp human thymidine kinase promoter described by Lipson et al. [4]. The −83 and −63 bp promoters were prepared from the latter by truncation at the −83 bp *Eag*I and −63 bp *Nco*I restriction sites respectively. Portions of the basic promoter between −30 and −48 bp or between −48 and −83 bp were excised and the remaining portions rejoined to yield plasmids lacking the proximal (ΔP) and distal (ΔD) CCAAT sites. PM, DM and PDM contained mutations of the CCAAT sites involving conversion of ATTGGCC into ATCAGCC at one or both of the proximal and distal sites in the −1210 promoter. In one construct the thymidine kinase promoter was replaced by the SV40 early promoter and in this plasmid the coding sequence was derived from the human thymidine kinase cDNA rather than the minigene. The shaded bar represents the start of the coding region.

Abbreviations used: SV, simian virus; DTPA, diethylenetriaminepenta-acetic acid.

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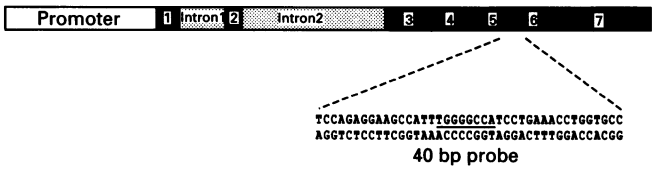

Figure 2 Structure of the −1210TK minigene with enlargement of the exon 5/6 boundary

The region underlined shows close identity with the Zn^{2+}-dependent zone of the human thymidine kinase promoter.

periment consisted of four treatments, a control group and three that received the metal chelator diethylenetriaminepenta-acetic acid (DTPA; 600 μM) to restrict the availability of Zn^{2+} during the final 24 h before harvesting the cells. Preliminary experiments indicated that, although Fe^{2+} was unable to replace Zn^{2+}, supplementation of the DTPA with Zn^{2+} and Fe^{2+} gave higher thymidine kinase activities than addition of Zn^{2+} alone, and therefore Fe^{2+} (200 μM) was always added along with the DTPA. One of the three DTPA-treated groups (−Zn) received no further supplementation and the other two received 200 or 400 μM Zn^{2+}. Triplicates of each treatment were prepared, harvested and assayed for thymidine kinase activity as previously described [1].

Gel-mobility-shift assays

The effects of Zn^{2+} availability on protein binding to the thymidine kinase promoter were investigated with BHK TK^- cells transfected with the human thymidine kinase minigene construct driven by the −1210 bp promoter. Cells grown in control medium were compared with those transferred for the final 24 h to medium containing DTPA/Fe^{2+} (600/200 μM) either alone or in combination with either 200 or 300 μM Zn^{2+}. In order to compare the growth check induced by lack of Zn^{2+} with that caused by serum starvation, the cells used in one experiment were rendered quiescent by exposure for 72 h to serum-free medium. Cells in a further group were synchronized at the beginning of S-phase by exposure to 0.5 mM hydroxyurea for 24 h before harvesting.

Gel-mobility-shift assays were based largely on those of Kim and Lee [5] using the method of Manley et al. [6] to prepare the cell extracts. Briefly, harvested cells were suspended in 4 cell vol. of hypotonic buffer (10 mM Tris/HCl, pH 7.9, 2 mM dithiothreitol, 0.2 mM PMSF) for 30 min at 0 °C then lysed by five passages through a 25-gauge needle. Then 4 cell vol. of extraction buffer [50 mM Tris/HCl, pH 7.9, 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 mM PMSF, 25% (w/v) sucrose, 50% (v/v) glycerol] and 1 vol. of satd. $(NH_4)_2SO_4$ were added and the mixture was stirred gently for 20 min before centrifugation at 250000 *g* for 3 h at 4 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant (0.3 g/ml) and after 30 min the precipitate was collected by centrifugation at 17000 *g* for 30 min. The pellet was dissolved in storage buffer [25 mM Hepes, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mM PMSF, 17% (v/v) glycerol] and dialysed against two changes of the same buffer over a period of 16 h. The preparation was then stored frozen in small aliquots at −80 °C. Before assay, the protein concentration of the extract was determined by the method of Lowry et al. [7], and

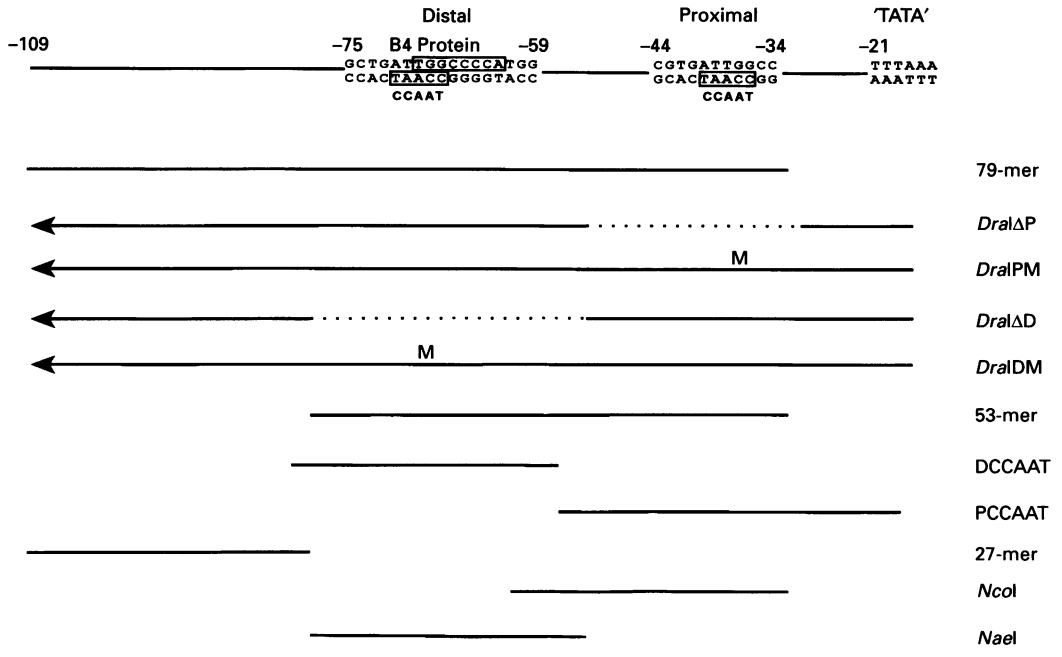

Figure 3 Diagrammatic representation of the oligonucleotides used to compete with the ^{32}P-labelled 79 bp probe for binding of proteins from extracts of −1210TK cells

The upper line illustrates the structure of a proximal portion of the human thymidine kinase gene including the two CCAAT and the band-4 protein-binding sites. The numbers indicate base pairs proximal from the transcription initiation site. The 79-mer was a non-radioactive version of the full probe. ΔP, PM, ΔD and DM and PDM were fragments excised from the corresponding plasmids shown in Figure 1 by restriction with *Dra*I at −20 and −241 bp with respect to the unmodified sequence. The arrows indicate that the oligonucleotides extended beyond the region illustrated to −241 bp without modification to the gene sequence. A similar fragment (not shown) was obtained from the unmodified −1210 bp promoter. The 53-mer resulted from Klenow extension of a 21 bp oligonucleotide hybridized to the 3′ end of the proximal 55 bp oligonucleotide used to synthesize the probe. The 53 bp oligonucleotide was also restricted with *Nco*I or *Nae*I to yield the oligonucleotides so labelled. The PCCAAT and DCCAAT competitors were prepared by annealing the corresponding complementary oligonucleotides. Finally, the 27-mer corresponded to the distal section of the probe from −83 to −109 bp.

Table 1 Influence of promoter structure on the expression of human thymidine kinase activity from plasmids containing a human thymidine kinase minigene construct

The promoters activating the human thymidine kinase plasmids were those described in Figure 1. They were used to activate the human thymidine kinase minigene except for the SV40 promoter which was attached to the human thymidine kinase cDNA. The results represent means for six replicates per treatment and the standard errors of the differences were calculated from the pooled S.D. obtained by analysis of variance of the data for individual plasmids. nd, Not detected.

Transfected plasmid	Control activity (pmol/min per mg of protein)	Thymidine kinase activity (% of activity in control cells)			S.E. of the differences between mean percentages
		+ DTPA/Fe^{2+}	+ DTPA/Fe^{2+}/ 200 μM Zn^{2+}	+ DTPA/Fe^{2+}/ 400 μM Zn^{2+}	
−1210TKi	72	10	45	48	8
−83TKi	22	1	46	121	4
−63TKi	3	nd	62	83	11
−1210TKiΔP	5	42	156	194	12
−1210TKiPM	8	19	101	124	13
−1210TKiΔD	14	6	87	61	10
−1210TKiDM	10	3	54	61	18
−1210TKiPDM	18	3	78	72	8
SV40TKc	13	36	63	43	13

the extract diluted with storage buffer to a final concentration of 2 mg of protein/ml.

For the gel-mobility-shift assays, the standard binding mixture (20 μl) contained 1 μl of ^{32}P-probe, 2 μl of cell extract, 10 mM Tris/HCl, pH 7.5, 2 μg dIdC, 1 mM dithiothreitol and 50 mM NaCl. The mixture was incubated at room temperature for 30 min and then 5 μl of 50% (v/v) glycerol/0.1% Bromophenol Blue in 10 mM Tris/HCl buffer, pH 7.5, was added. Aliquots of volume 12 μl were loaded on duplicate gels containing 6% (w/v) acrylamide (1/80th bis). The gels were pre-electrophoresed in 190 mM glycine/25 mM Tris/HCl, pH 7.5, at 4 °C and 7.4 mA/gel for 1 h and run for a further 1.75 h after loading of the samples. The gels were then soaked in 10% (v/v) acetic acid for 10 min before drying. The bands of radioactivity in the gels were localized and quantified with a micro-channel array detector (Instant Imager, Canberra Packard). This provided a high-resolution two-dimensional quantitative image of the blot which was analysed to determine the radioactivity associated with the individual bands.

For most experiments, the probe contained the sequence of the human thymidine kinase gene between −109 and −31 bp upstream of the transcription start site (numbering according to Arcot et al. [8]) and it was synthesized and labelled with [^{32}P]dCTP by Klenow extension of two overlapping 55 bp oligonucleotides. Each reaction mixture contained approx. 2 ng of probe in the final volume of 20 μl equivalent to a final concentration of approx. 2 nM. For certain experiments, a ^{32}P-labelled 40 bp probe with a sequence overlapping the boundary between the fifth and sixth exons in the human thymidine kinase cDNA (Figure 2) was synthesized by extension of overlapping 9 bp and 40 bp oligonucleotides. Before use, the probes were purified by electrophoresis through acrylamide gels.

In order to determine the DNA-binding specificity of the observed complexes, a range of non-radioactive probes was prepared to act as competitors (Figure 3). Several of these were obtained by *DraI* restriction of the modified plasmids used to transfect cells for thymidine kinase activity measurements. This resulted in a fragment located between −241 and −20 bp of the control sequence. Other competitors were obtained by Klenow extension of the individual 55 bp oligonucleotides to yield 55 bp duplexes and then further processing of these with restriction enzymes. In all cases, PAGE was used to purify and confirm the identity of the products. Oligonucleotides containing the consensus binding sequence for the SP1 and NF-1 transcription factors (Promega, Southampton, Hants., U.K.) were used as non-specific competitors in the gel-mobility-shift assays.

RESULTS

Table 1 illustrates the influence of Zn^{2+} availability on the expression of thymidine kinase activity from plasmids containing modifications of the human thymidine kinase promoter. Compared with the −1210TKi plasmid, each of the modifications to the promoter decreased the thymidine kinase activity observed in control cells. Despite this all were able to replicate at comparable rates in media containing HAT which inhibits the synthesis *de novo* of deoxythymidine monophosphate. Thymidine kinase activity in each of the cell lines was substantially lower when the availability of Zn^{2+} was restricted by the addition of DTPA. However, the extent of inhibition was less marked with the SV40 promoter than with most of the thymidine kinase promoters, suggesting that the latter were specifically sensitive to lack of Zn^{2+}. In contrast, the thymidine kinase promoters lacking the proximal CCAAT-binding site either as a result of deletion (ΔP) or mutation (PM) also appeared to be less sensitive to Zn^{2+} deprivation. Despite this, the promoter with a mutation at both proximal and distal CCAAT sites was fully sensitive to low Zn^{2+} availability. These observations suggested that the sensitivity of thymidine kinase activity to lack of Zn^{2+} was at least partially dependent on the nature of the thymidine kinase promoter but failed to provide a clear indication of the basis for this effect.

In order to clarify the role of the promoter in the dependence of thymidine kinase activity on Zn^{2+} supply, a labelled probe was prepared containing the sequence of the human thymidine kinase promoter between −109 and −31 bp relative to the transcription initiation site. This zone has been reported to contain most of the regions thought to regulate the activity of the human thymidine kinase promoter [4,5,8,9]. Furthermore, both previous studies [2] and the present results indicated that even when the thymidine kinase promoter was truncated to −83 bp, thymidine kinase activity remained fully sensitive to lack of Zn^{2+}, suggesting that the region between 0 and −83 bp was likely to contain any Zn^{2+}-sensitive elements present within the promoter. When the probe was used in gel-mobility-shift assays, six distinct retardation

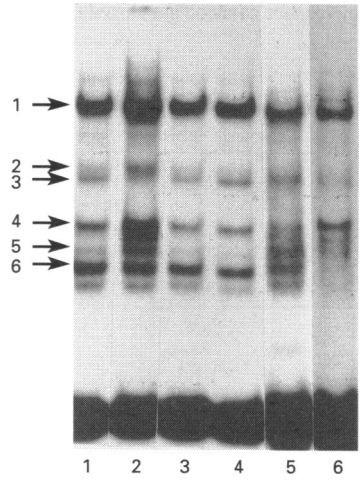

Figure 4 Effects of growth restriction on band patterns in gel-mobility-shift assays

A ^{32}P-labelled probe corresponding to the region between −31 and −109 bp of the human thymidine kinase promoter was incubated with cell extracts from control cells (lane 1) or cells treated with DTPA/Fe^{2+} (lane 2), DTPA/Fe^{2+} + 200 μM Zn^{2+} (lane 3) or DTPA/Fe^{2+} + 300 μM Zn^{2+} (lane 4). Further extracts were obtained from cells after exposure to serum-free medium for 72 h (lane 5) and after exposure to 500 μM hydroxyurea for 24 h (lane 6). After equilibration of the extracts with the probe, the mixtures were separated on a non-denaturing acrylamide gel; the gels were then dried and autoradiogaphed. The arrows mark the position of the bands referred to in the text.

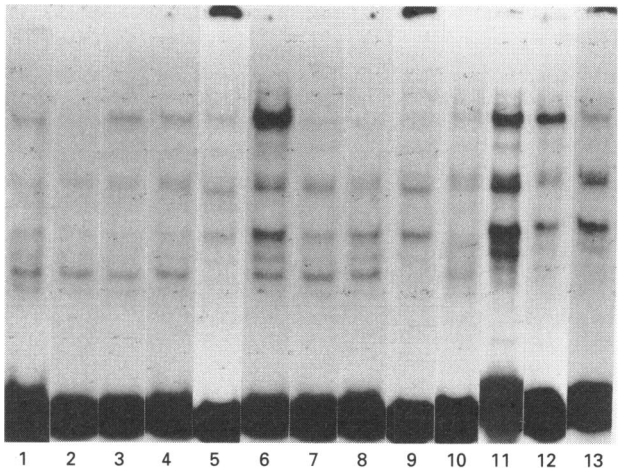

Figure 5 Effects of competitor oligonucleotides on the binding of proteins from control cell extracts to the ^{32}P-labelled 79-mer probe in gel-mobility-shift assays

The probe was incubated with cell extracts from control cells in the presence of competitor oligonucleotides (Figure 3). The following oligonucleotides were used individually in the lanes indicated: 1, non-radioactive 79-mer; 2, *Dra*I control; 3, *Dra*IΔP; 4, *Dra*IPM; 5, DCCAAT; 6, control without competitor; 7, *Dra*IΔD; 8, *Dra*IDM; 9, PCCAAT; 10, 53-mer; 11, 27-mer; 12, *Nae*I; 13, *Nco*I. After equilibration of the extracts with the probe, the mixtures were separated on a non-denaturing acrylamide gel; the gels were then dried and autoradiographed.

bands were observed although bands 2 and 3 were not well resolved (Figure 4). A number of other minor bands were also observed with certain preparations but these were less consistent than those numbered and were ignored in subsequent analyses. Despite equal quantities of total protein being applied to each lane of the gel, there was a twofold increase in the proportion of the total counts associated with band 4 when the extracts were prepared from cells exposed to DTPA for the previous 24 h (Table 2). There was also a significant increase in the proportion of the total counts associated with band 5. However, when the counts in the individual bands were expressed relative to the total counts bound, the predominant effect was a substantial elevation of the proportion associated with band 4. Supplementation of the DTPA-treated cultures with either 200 or 300 μM Zn^{2+} completely reversed these effects. As lack of Zn^{2+} restricts the growth of cells, the altered binding pattern could have been a consequence of the slowing of growth rather than the result of Zn^{2+} loss. However, the proportion of the activity present in band 4 was not increased when growth of the cells was inhibited by serum deprivation or exposure to hydroxyurea (Figure 4, Table 2).

In order to ascertain the DNA-binding specificity of the band-4 complex, a series of gel-mobility-shift assays was performed in which the binding of the nuclear proteins to the labelled 79 bp probe was competed for by addition of one of a range of unlabelled oligonucleotides with related base sequences (Figures 3 and 5). A series of concentrations of each competitor was investigated and from these the concentration required to inhibit the uptake of the labelled probe by 50% (IC_{50}) was estimated for each complex (Table 3). From these experiments it became apparent that oligonucleotides containing the sequence between −83 and −55 bp competed for the band-4 complex more

Table 2 Distribution of radioactivity between bands in gel-mobility-shift assays of BHK −1210TKi cell extracts probed with a ^{32}P-labelled 79 bp oligonucleotide from the human thymidine kinase promoter

The experimental treatments were as described in the Experimental section. Values are means ± S.E.M. for number of replicates indicated.

Group	No. of replicates	Percentage of total counts						Percentage of total bound counts					
		Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6
Control	12	8.28 ± 0.56	1.14 ± 0.10	1.14 ± 0.13	3.65 ± 0.31	1.58 ± 0.12	1.48 ± 0.21	47.9 ± 1.1	6.6 ± 0.5	6.5 ± 0.5	21.0 ± 1.0	9.2 ± 0.6	8.8 ± 1.3
DTPA/Fe^{2+} (−Zn)	8	10.21 ± 1.35	1.57 ± 0.10	1.34 ± 0.14	8.61 ± 0.57	2.61 ± 0.14	1.79 ± 0.42	38.2 ± 2.0	6.2 ± 0.4	5.1 ± 0.2	33.9 ± 2.6	10.3 ± 0.9	6.4 ± 1.1
DTPA/Fe^{2+}/ 200 μM Zn^{2+}	8	8.17 ± 0.,55	1.08 ± 0.12	0.99 ± 0.06	4.05 ± 0.57	1.39 ± 0.08	1.51 ± 0.23	47.3 ± 0.9	6.2 ± 0.5	6.0 ± 0.6	23.0 ± 2.0	8.3 ± 0.7	9.2 ± 1.7
DTPA/Fe^{2+}/ 300 μM Zn^{2+}	8	9.15 ± 0.68	1.13 ± 0.13	1.32 ± 0.08	3.52 ± 0.44	1.21 ± 0.11	1.59 ± 0.20	50.8 ± 1.4	6.3 ± 0.5	7.5 ± 0.5	19.3 ± 1.4	6.8 ± 0.6	9.3 ± 1.5
Quiescent	4	2.73 ± 0.54	0.49 ± 0.16	0.77 ± 0.18	1.91 ± 0.58	1.41 ± 0.24	1.11 ± 0.24	32.2 ± 1.0	5.5 ± 0.9	8.9 ± 0.9	22.3 ± 1.1	16.8 ± 0.4	14.3 ± 2.7
Hydroxyurea	4	2.11 ± 0.11	0.28 ± 0.02	0.28 ± 0.02	1.28 ± 0.08	0.93 ± 0.09	0.50 ± 0.02	39.2 ± 1.9	5.1 ± 0.2	5.2 ± 0.3	23.8 ± 0.8	17.4 ± 1.8	9.3 ± 0.1

Table 3 Competition by non-radioactive oligonucleotides for binding of proteins from extracts of BHK −1210TKi cells to a ^{32}P-labelled 79 bp oligonucleotide from the human thymidine kinase promoter

The competitor oligonucleotides used in these experiments were those described in Figure 3. Several concentrations of each competitor were investigated and the IC_{50} in each band was estimated.

Competitor	IC_{50} (nM)					
	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6
Unlabelled 79 bp probe	5	12	14	12	15	14
*Dra*I Control	1	16	16	8	> 50	> 50
*Dra*IΔP	8	4	4	4	> 50	> 50
*Dra*IPM	10	12	16	12	33	> 50
DCCAAT	2	> 50	> 50	10	18	27
*Dra*IΔD	5	18	18	32	> 50	> 50
*Dra*IDM	2	18	18	31	42	42
PCCAAT	1	40	> 50	27	27	40
53-mer	8	12	12	9	50	> 50
27-mer	27	27	45	> 50	> 50	> 50
*Nae*I	25	6	13	9	21	21
*Nco*I	5	> 50	> 50	> 50	> 50	> 50

effectively than those that lacked this region of the promoter or in which the TTGG sequence at −70 bp had been mutated to TCAG. Two unrelated oligonucleotides containing consensus sequences for SP1 and NF-1 transcription factors failed to compete with any of the numbered bands (Figure 6).

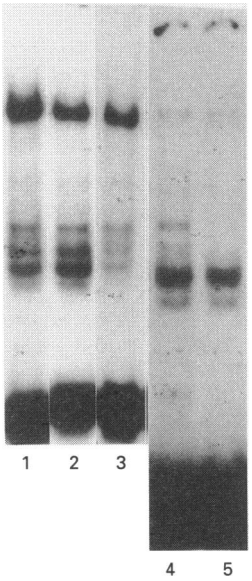

Figure 6 Banding patterns obtained with control extracts in gel-mobility-shift assays using the 79-mer probe from the human thymidine kinase promoter and a 40-mer probe spanning the exon 5/6 boundary of the thymidine kinase cDNA

The nature and location of the 40-mer probe sequence is illustrated in Figure 2. The control cell extracts were incubated with radioactive probes and competitor oligonucleotides and loaded into the lanes as follows: 1, 79-mer probe only; 2, 79-mer probe + SP1 oligonucleotide; 3, 79-mer probe + NF-1 oligonucleotide; 4, 40-mer probe only; 5, 40-mer probe + non-radioactive 79-mer. After equilibration of the extracts with the probe, the mixtures were separated on a non-denaturing acrylamide gel; the gels were then dried and autoradiographed.

DISCUSSION

Inhibition of thymidine kinase activity by DTPA is specifically reversible by addition of Zn^{2+} [10–12], the effects of the chelator on thymidine kinase activity being associated with comparable changes in thymidine kinase mRNA [1,2]. The presence of Zn^{2+} in a wide range of transcription factors, the 'zinc-finger' proteins, suggested that the sensitivity of thymidine kinase mRNA to Zn^{2+} availability might be mediated through binding of Zn^{2+}-dependent proteins to the thymidine kinase promoter. Furthermore, the significantly greater inhibition of thymidine kinase activity in DTPA-treated cultures when the thymidine kinase coding region was under the control of its own promoter rather than that of the SV40 early promoter further suggested that the human thymidine kinase promoter was specifically sensitive to Zn^{2+} deprivation. This was not a consequence of the SV40 promoter being linked to the thymidine kinase cDNA sequence rather than to the minigene coding region used in the other constructs, as a construct with the −1210 TK promoter driving the thymidine kinase cDNA was also fully sensitive to lack of Zn^{2+} (results not shown).

Each of the modifications to the human thymidine kinase promoter significantly decreased its strength. This was consistent with the presence of a number of known sites of transcriptional activation within the regions excised or modified [4,5,8,9]. However, despite these variations in intrinsic promoter strength, all constructs remained at least partially sensitive to lack of Zn^{2+}. When the human thymidine kinase promoter was truncated to just 63 bp 5′ to the transcription start site, the construct remained fully sensitive to Zn^{2+}. The human thymidine kinase promoter contains two CCAAT protein-binding sites in reverse orientation at −40 and −71 bp which have been implicated in the activation of thymidine kinase transcription [4,8,9]. Removal or mutation of the more proximal CCAAT site at −40 bp rendered the thymidine kinase construct significantly less sensitive to lack of Zn^{2+}. This suggested a possible involvement of the proximal CCAAT site in the sensitivity of the thymidine kinase promoters to Zn^{2+}. However, the known CCAAT-binding proteins do not contain zinc-fingers and the double mutant with modifications to both proximal and distal CCAAT sites was fully sensitive to Zn^{2+} deprivation. The results of these transfection experiments seemed to confuse rather than clarify the involvement of the thymidine kinase promoter in the response of thymidine kinase activity to Zn^{2+} availability.

When the region of the human thymidine kinase promoter between −109 and −31 bp was used as a probe in gel-mobility-shift investigations, six retardation bands were observed but only one, band 4, showed an unambiguous response to the Zn^{2+} status of the cultures. As most zinc-finger proteins appear to act as activators of transcriptions, the initial hypothesis had predicted that loss of promoter activity in Zn^{2+}-deficient extracts would result from loss of such an activator in the absence of adequate Zn^{2+}. In contrast, the results clearly indicated that the lower activity of thymidine kinase in DTPA-treated cultures was associated with a significant and specific increase in the radioactivity in band 4. Although the quantities of protein in this fraction were too low to be detectable on stained gels, the increase in counts presumably resulted from an increase in the protein present in band 4. Furthermore, as lack of Zn^{2+} is known to depress the thymidine kinase mRNA content of cells but increased the concentration of the band-4 protein, it is unlikely that this protein acts as a transcriptional activator of thymidine kinase. Moreover, neither addition of DTPA nor of Zn^{2+} to the mobility-shift assay *in vitro* influenced the binding patterns observed (results not shown). The elevated concentrations of the

band-4 protein in Zn^{2+}-deficient cells, its possible function as an inhibitor rather than as an activator of transcription and its insensitivity to Zn^{2+} supply *in vitro* all suggest that it is unlikely that band 4 contains a zinc-finger protein.

The competition between unlabelled oligonucleotides and the labelled 79 bp probe indicated that the binding site for the band-4 protein lay between −55 and −83 bp and included the nucleotides at −69 and −70 pb, as mutation of these markedly altered the ability of the oligonucleotides to compete for the band-4 protein. A protein complex similar to that in band 4 has been observed in independent experiments with several types of cells [13]. Studies with HepG2 cell nuclear extracts suggested that the protein that forms this complex may bind to the partially palindromic sequence TGGCCCCA lying between −62 and −69 bp of the human thymidine kinase promoter [13].

Although there is no direct evidence that the band-4 protein inhibits transcription of the thymidine kinase gene, it is noteworthy that the decreased thymidine kinase concentration repeatedly observed in Zn^{2+}-deficient cells was here associated with increased concentration of the band-4 protein and that its apparent binding site overlaps the distal CCAAT site. Thus the elevated concentration of band-4 protein observed in the extracts from Zn^{2+}-deficient cells could compete with the CCAAT-binding protein and lower the latter's ability to bind to the thymidine kinase promoter. As the CCAAT-binding proteins have been shown to activate the thymidine kinase promoter [4,5,8], their displacement from it by excess band-4 protein could account for an inhibitory effect of the latter.

Comparison with the studies of Arcot et al. [8], Chang and Liu [14] and Lipson et al. [13] suggests that band 1 contained the CCAAT-binding protein NF-Y. In the gel-mobility-shift assays, binding sites on the probe were in large excess over the available binding proteins yet the numbers of molecules associated with B1 were only twice those bound to B4 (cf. the relative counts in bands 1 and 4 in Table 2). As there are two CCAAT-binding sites for each B4 site, there is likely to be competition between band-1 and band-4 proteins for binding to the distal CCAAT site. If the band-4 protein did inhibit transcription of thymidine kinase, this competition between the two proteins would provide a rationale for the loss of Zn^{2+} sensitivity observed when the proximal CCAAT site was modified or excised. The latter is known to have a higher affinity for the CCAAT protein than the distal site and would thus be expected to be occupied preferentially [8,9]. However, with those plasmids lacking a functional proximal site, additional CCAAT-binding protein would be available at the distal site thereby reducing the ability of the band-4 protein to compete for binding to the overlapping region. One would thus expect mutation or excision of the proximal CCAAT site to reduce the sensitivity of the promoter to the elevated concentrations of band-4 protein found in Zn^{2+}-deficient cells. This agrees with the observed loss of sensitivity of thymidine kinase activity to Zn^{2+} deprivation in the cells transfected with the ΔP and PM constructs. Note that this explanation relies on competition between the two binding proteins at the distal site which would not occur when mutation of the latter abolished its affinity for the CCAAT-binding protein. The failure of the proximal mutation to decrease the Zn^{2+} sensitivity of the PDM construct which also lacked a functional distal CCAAT site was not therefore anomalous.

The above explanation for the Zn^{2+} effect on the thymidine kinase promoter would suggest that the constructs lacking the putative TGGCCCCA-binding site for the band-4 protein might be relatively insensitive to Zn^{2+} deprivation. However, they also lacked a functional distal CCAAT site. Any potential increase in the strength of the promoter associated with loss of the postulated inhibitory effect of the band-4 protein would therefore be at least partially countered by the concomitant loss of activation of the promoter at the distal CCAAT site. In practice, each of the constructs lacking the TGGCCCA site in their promoter was still fully sensitive to lack of Zn^{2+}. This led to a re-examination of the human thymidine kinase gene and the recognition of an identical TGGCCCCA sequence in reverse orientation within the sixth exon. Thus the most likely explanation for the sustained sensitivity to Zn^{2+} of the constructs lacking the critical sequence in their promoter was that the band-4 protein also binds to this sequence within the sixth exon. In the absence of adequate Zn^{2+}, binding of increased quantities of band-4 protein within the sixth exon could result in transcriptional pausing. Two experimental observations support this hypothesis. First, gel-mobility-shift assays were performed (Figure 6) using a synthetic oligonucleotide probe with a sequence that spanned that of the TGGCCCCA-binding site in the sixth exon (Figure 2). These demonstrated the formation of a complex with the proteins of the cell extract that had a mobility very similar to that of the band-4 promoter complex. Secondly, the non-radioactive form of the promoter probe competed strongly (50% inhibition at 6 nM) with the radioactive form of the 40 bp probe for binding to the band-4 protein.

Although there is at present no direct evidence for an inhibitory effect of the band-4 protein, competition with the CCAAT activator for attachment to overlapping binding sites would provide a rational basis for such an effect which could underlie at least part of the sensitivity of the human thymidine kinase promoter to lack of Zn^{2+}. The nature of the protein and its potential ability to induce transcriptional pausing are currently under investigation.

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