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Protein-DNA Interactions Associated with the Onset of Testis-Specific Expression of the Mammalian *Pgk-2* Gene

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We have identified differences in protein-DNA interactions associated with the promoter of the mammalian spermatogenesis-specific *Pgk-2* gene in expressing and nonexpressing cells, using a band shift assay. We compared DNA-binding activities in nuclear protein extracts from expressing adult testis cells versus nonexpressing prepuberal testis cells and nonexpressing somatic cells. One or two DNA-binding activities were found to be uniquely associated with the expressed state of *Pgk-2*, while a third appears to be associated with the nonexpressed state. All three of these activities map to a region within the first 40 bp upstream from the core promoter of this gene. The *Pgk-2* core promoter lacks a TATA box but contains a GC box and a CAAT box. We show that the GC box binds the ubiquitous transcription factor Sp1 and that the CAAT box binds CTF-1, both of which are present in extracts from all three tissue types tested. These results suggest that tissue-specific transcription of the *Pgk-2* gene is associated with changes in protein-DNA interactions occurring within a 40-bp enhancer region and that different arrays of protein-DNA interactions in this region are associated with the actively expressed state of the *Pgk-2* gene in spermatocytes and spermatids and with the terminally repressed state of *Pgk-2* in somatic cells.

Phosphoglycerate kinase is a key enzyme involved in the metabolism of glucose or fructose in glycolysis. In mammals, phosphoglycerate kinase is encoded by two functional genes, *Pgk-1* and *Pgk-2* (5, 29, 30). *Pgk-1* is X linked and is ubiquitously expressed at relatively low levels in all somatic cells, oogenic cells, and premeiotic spermatogenic cells (reviewed by VandeBerg [38]). *Pgk-2* is autosomal and is expressed as a tissue-specific gene at high levels during spermatogenesis, specifically in meiotic spermatocytes (preleptotene through pachytene) and postmeiotic spermatids (steps 1 to 9) (23, 39). Spermatogenesis represents a model developmental system in mammals, and the cell type- and stage-specific expression of the *Pgk-2* gene within this system provides an opportunity to study the regulation of differential gene expression during the development of a particular cell lineage.

Previous studies showed that a 327-bp sequence in the 5′ regulatory region of the human *Pgk-2* gene was required to direct testis-specific expression of reporter genes in transgenic mice (32). This tissue-specific enhancer region lies immediately upstream from the core promoter in the human *Pgk-2* gene (Fig. 1). The *Pgk-2* core promoter is a region approximately 190 bp in length that contains sequence motifs common to many mammalian gene promoters, but it was shown to be insufficient on its own to direct testis-specific expression in transgenic mice (32).

The *Pgk-2* core promoter lacks a TATA-box homology but contains a GC box at the position where a TATA box would normally be found (−26 to −32 bp upstream from the single transcription start point) and a CAAT box further upstream at −97 to −102 (27). GC-box sequences in other gene promoters have been shown to bind the transcription factor Sp1 (10–14). Several proteins that specifically bind CAAT-box sequences, including the CTF family of polypeptides (34), have been described (2, 9, 15, 16, 19, 20). Sp1 and CTF have been found in a wide variety of somatic cell types (18, 33) and are thus not likely to act as primary regulators of tissue-specific gene expression. They have, however, been invoked as regulators of the location or frequency of initiation of transcription from tissue-specific genes in somatic cells (34, 35), and they may also act to mediate effects of certain tissue-specific transcription factors in these cells (25, 31). Although putative binding sites for one or both of these ubiquitous factors have been identified in promoter sequences from other genes expressed in mammalian spermatogenic cells (17, 22), their ability to bind to sequences in the promoter of a mammalian spermatogenesis-specific gene has not been demonstrated. Thus, in the first part of this study, we investigated the capacity of the *Pgk-2* core promoter, especially the GC and CAAT boxes, to bind nuclear proteins from testicular as well as somatic cells.

Tissue-specific gene expression in somatic cells is typically regulated, at least in part, by protein-DNA interactions, and there is no reason to assume this is not the case for spermatogenic cell-specific gene expression as well. However, no specific protein-DNA interaction has yet been correlated with the onset of transcription of a particular gene during spermatogenesis in mammals. In the absence of a reliable transient expression assay system for mammalian spermatogenic cells in which to test numerous promoter deletions, we focused on the 327-bp *Pgk-2* enhancer region previously identified by experiments with transgenic mice (32) in our search for one or more protein-DNA interactions associated with the onset of *Pgk-2* transcription. Initially, we compared the capacity of nuclear protein extracts from adult testis cells (in which *Pgk-2* is expressed) with that of nuclear or whole cell protein extracts from somatic cells (in which *Pgk-2* is not expressed) to generate specific protein-DNA

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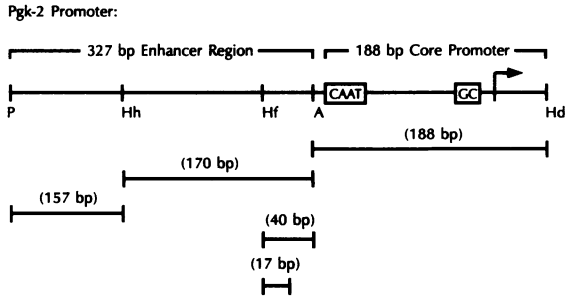

FIG. 1. Schematic representation of the *Pgk-2* promoter depicting the core promoter, which lacks a TATA-box sequence but possesses a single GC-box sequence at −26 to −32 bp upstream from a single transcription start point (27) and a single CAAT-box sequence at −96 to −100, and the enhancer region immediately 5′ to the core promoter. The enhancer region was shown to contain sequences required to direct testis-specific expression of reporter genes in transgenic mice (32). The locations of the *Pgk-2* promoter fragments used as probes in the band shift experiments are shown. These fragments were delineated by restriction endonuclease sites: P (*Pst*I), Hh (*Hha*I), Hf (*Hin*fI), A (*Ava*II), and Hd (*Hin*dIII).

interactions in the *Pgk-2* enhancer region. Since the onset of *Pgk-2* expression occurs at puberty, we then compared the interaction of sequences in the *Pgk-2* enhancer region with nuclear proteins from nonexpressing prepuberal testicular cells versus expressing adult testicular cells.

We report here that both prepuberal and adult testicular cells contain the ubiquitous transcription factors Sp1 and CTF and that these factors bind in a sequence-specific manner to the GC and CAAT boxes in the *Pgk-2* core promoter. In addition, we show that different, specific protein-DNA interactions in the *Pgk-2* enhancer region are correlated with the expressed and nonexpressed states of this gene, respectively. A model is presented to describe the manner in which these enhancer-binding activities change in association with the onset of *Pgk-2* transcription during spermatogenesis.

MATERIALS AND METHODS

Reagents. Restriction enzymes and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories or Boehringer Mannheim. Protease inhibitors were purchased from Sigma. Poly(dI-dC) · (dI-dC) was purchased from Pharmacia. Antibody to Sp1 was a rabbit polyclonal antibody that was generously provided by S. Jackson and R. Tjian. CTF-1 protein was kindly provided by N. Tanese and R. Tjian. This protein was generated by a recombinant vaccinia virus (V1) and was purified from infected HeLa cells to homogeneity by affinity chromatography (36).

Preparation of DNA fragments and double-stranded oligomers. DNA fragments for use in the band shift assay were prepared by the polymerase chain reaction (PCR). The template used in all cases was a PCR-generated *Xba*I-*Eco*RI fragment of Pgk-2/CAT (32) that included the *Pgk-2* promoter region. After amplification using the appropriate oligonucleotides as primers, each fragment was purified by phenol-chloroform extraction and ethanol precipitation. The amplified product was resuspended in 1× Tris-EDTA (pH 7.4), and the ragged ends were filled in with Klenow enzyme and a 2 mM concentration of each of the four deoxynucleoside triphosphates to yield blunt-ended fragments. These fragments were digested with the appropriate restriction endonucleases to release the required subfragments, which were then separated in 1.5 to 2.5% agarose–5% Nusieve GTG agarose, electrophoresed onto NA45 membranes (Schleicher & Schuell), and purified as described previously (1).

High-pressure liquid chromatography-purified oligonucleotides were obtained from the Johns Hopkins DNA synthesis core facility and annealed by heating to 60 to 65°C for 5 min and cooling slowly to room temperature. Double-stranded oligonucleotides were concatemerized by first phosphorylating with T4 polynucleotide kinase and then ligating with T4 DNA ligase. The GC-box-containing, double-stranded oligonucleotide was purchased from Stratagene.

Extract preparation. HeLa whole cell extracts (26) and HeLa nuclear extracts (8) were purchased from Stratagene. Testis nuclear extracts were prepared as described by Bunick et al. (7), with some modifications. Testes from 50 adult (>60 days old) and 240 5-day-old CD-1 mice (Charles River) were dissected and decapsulated. The 5-day testes were homogenized directly; the adult testes were finely minced prior to homogenization. Homogenization was done according to the method of Bunick et al. (7) except that a battery of protease inhibitors (leupeptin [1 μg/ml], aprotinin [2 mg/ml], ovalbumin [2 mg/ml], and phenylmethylsulfonyl fluoride [0.1 mM]) was added to the homogenization buffer and at lesser concentrations (0.01 μg/ml, 20 μg/ml, 20 μg/ml, and 0.1 mM, respectively) to the dialysis buffer. Dialysis was performed in 0.5-ml microcollodion bags (Sartorius SM 13202) for 2 to 2.5 h with a change of buffer after 1 h. Protein concentrations were measured by optical absorbance (21) and were consistently 15 to 16 mg/ml. Extracts were aliquoted and stored in liquid N_2 until use.

Band shift assay. DNA probes were prepared by end filling the appropriate DNA fragment or double-stranded oligonucleotide (Fig. 1) with Klenow DNA polymerase and either [α-^{32}P]dGTP or [α-^{32}P]dCTP and were then purified on Sephadex G-50. Binding reactions were carried out in 10% glycerol–25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9)–50 mM KCl–0.5 mM EDTA–0.5 mM dithiothreitol–0.05 mM phenylmethylsulfonyl fluoride. Protein samples (1 μg of HeLa extract or 15 μg of testis extract) were added to the reaction mix in the presence of poly(dI-dC) · (dI-dC) made up in 100 mM NaCl: 0.8 μg for HeLa cell extract, 1.2 μg for HeLa nuclear extract, 5 μg for adult testis extract, and 7.5 μg for 5-day testis extract. Where appropriate, unlabeled DNA competitor fragments or Sp1 antibodies were then added to the reaction mix. After addition of all components other than probe, the reaction mixes were incubated on ice for 5 min; then 5,000 to 20,000 cpm (0.3 to 0.5 ng) of end-labeled DNA probe was added to bring each reaction mix to a final volume of 10 μl, and the reaction mixes were incubated for another 20 min at 20°C (the binding reaction mix worked equally well when incubated at 0 or 30°C). After incubation, reactions were stopped by addition of 1 μl of 1% bromophenol blue, and resultant protein-DNA complexes were resolved by electrophoresis through 4% native polyacrylamide gels in low-ionic-strength buffer (50 mM Tris-HCl–1 mM EDTA [pH 8.0]). Gels were prerun at 30 mA and then run with samples at 60 mA for 1 to 4 h with recirculation of buffer, dried onto Whatman 3MM paper, and autoradiographed to Kodak XAR-5 film.

Band shift assays using 15 ng of purified CTF-1 protein instead of nuclear extract were performed as described above except that only 0.1 μg of poly(dI-dC) · (dI-dC) was

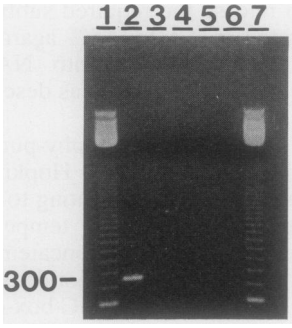

FIG. 2. rtPCR analysis of *Pgk-2* expression in tissues from which extracts were derived. Total RNAs (500 ng) from adult mouse testis (lane 2), prepuberal (5-day) mouse testis (lane 3), and HeLa cells (lane 4) were subjected to analysis by rtPCR, using a kit from Perkin Elmer according to the manufacturer's instructions. A no-template control (lane 5) and a no-reverse-transcriptase control (with adult mouse testis RNA) (lane 6) were also run. The products of these reactions were electrophoresed through 1% agarose and visualized by staining with ethidium bromide. A 300-bp amplimer was clearly detectable in the adult testis lane, but only very faint bands were seen in the prepuberal testis lane and the HeLa cell lane (the latter were equivalent to the faint band seen in the no-reverse-transcriptase control, suggesting this band is due to a very slight DNA contamination). The *Pgk-2* amplimer was sized by comparison with a 123-bp ladder (Bethesda Research Laboratories) (lanes 1 and 7).

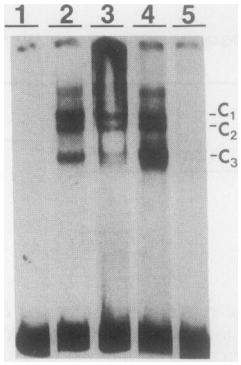

FIG. 3. Band shift assay on the 188-bp *Pgk-2* core promoter fragment. End-labeled *Pgk-2* core promoter fragment was incubated with no protein (lane 1), adult testis protein extract (lane 2), 5-day testis protein extract (lane 3), HeLa cell protein extract (lane 4), and adult testis protein extract plus excess (100×) unlabeled core promoter fragment (lane 5). Extracts from all three tissues produced the same three ubiquitous binding activities (C_1 to C_3), which were prevented by incubation with unlabeled fragment. A fourth band was occasionally visible just below band C_3 (e.g., see lane 4) and appeared to represent a degraded form of the activities represented in bands C_1 and C_2.

used in addition to 5 μg of bovine serum albumin in the binding reaction mix.

RESULTS

Tissue-specific transcription of *Pgk-2*. We investigated protein-DNA interactions associated with the *Pgk-2* promoter in three different tissues: testis from adult mice (≥60 days old), which contains the spermatogenic cell types expressing *Pgk-2*; testis from 5-day-old (prepuberal) mice, which lacks the spermatogenic cell types that express *Pgk-2*; and HeLa cells that represent a somatic cell type control in which *Pgk-2* is not expressed. Differential expression of *Pgk-2* in these tissues was confirmed by analysis of RNAs from each, using reverse transcriptase-mediated PCR (rtPCR) (Fig. 2). Thus, *Pgk-2* RNA was detected in RNA from adult testis but not in RNAs from either prepuberal testis or HeLa cells. (Similar results were obtained by Northern [RNA] blot hybridization of the human *Pgk-2*:3′-specific probe [29] to each of these RNA samples [data not shown].)

Analysis of regulatory sequences within the *Pgk-2* core promoter. The *Pgk-2* core promoter is defined as a 188-bp fragment that spans the *Ava*II-to-*Hin*dIII restriction sites, as shown in Fig. 1. This core promoter lacks a TATA box but contains a GC box at −26 to −32 and a CAAT box at −97 to −102. These sequences have been shown in somatic cells to bind ubiquitous transcription factors, including Sp1 and CTF, respectively. To determine whether these consensus sequences bind these same ubiquitous transcription factors in testicular cells, we carried out band shift assays on the core promoter region, using nuclear extracts made from testicular cells from adult mice, testicular cells from 5-day-old mice, and HeLa cell nuclear or whole cell extracts. Three specific bands (labeled C_1 to C_3 in Fig. 3) were consistently generated in each case, and these bands showed the same electrophoretic mobilities regardless of the source of the extract (Fig. 3, lanes 2 to 4). Additional bands seen in some cases were shown to represent nonspecific protein-DNA interactions (data not shown). All of the specific bands could be prevented by including in the reaction mix an excess of a cold DNA fragment (cold competitor) containing the entire *Pgk-2* core promoter fragment (Fig. 3, lane 5).

To map the sequences in the *Pgk-2* core promoter responsible for each of the protein-binding activities indicated by the band shifts, we performed competition experiments using excess cold DNA subfragments produced by restriction endonuclease digestion of the core promoter (subfragments I and III in Fig. 4A) or specific double-stranded oligonucleotides representing specific sequences within the core promoter (Fig. 4A, oligonucleotides II and IV). Bands C_1 and C_2 were competed for by subfragment I and by oligonucleotide II (Fig. 4B, lanes 3 and 4), indicating these bands represent the binding of one or more factors to the GC box. Band C_3 was competed for by subfragment III and by a concatamer of oligonucleotide IV (Fig. 4B, lanes 5 and 6), indicating that this band results from binding of a factor to the CAAT box. In contrast, competitor V (a concatemerized oligonucleotide representing a region adjacent to the CAAT-box sequence) did not compete for any of the three bands (Fig. 4B, lane 7), indicating all three bands represent specific protein-DNA interactions.

(i) Transcription factor Sp1 binds to the *Pgk-2* GC-box sequence in testicular cells. To determine whether the factor binding the *Pgk-2* GC box in testicular cells is the transcription factor Sp1, antibodies to Sp1 were incubated with the labeled *Pgk-2* core promoter fragment and adult testis extract in preparation for a band shift assay. These antibodies specifically diminished bands C_1 and C_2 (Fig. 5, lanes 4 and 5), whereas band shift reaction mixes incubated with preimmune serum without antibody showed no such effect (Fig. 5, lanes 2 and 3), indicating specific involvement of Sp1 in these protein-DNA interactions. Note that a typical double shift was not observed, since the *Pgk-2* core promoter–Sp1–Sp1 antibody complex thus formed was too large to enter the polyacrylamide gel. This result was confirmed by electrophoresis through an agarose gel (data not shown). The observation of two bands corresponding to Sp1 binding to the single GC box in the *Pgk-2* core promoter may be due to

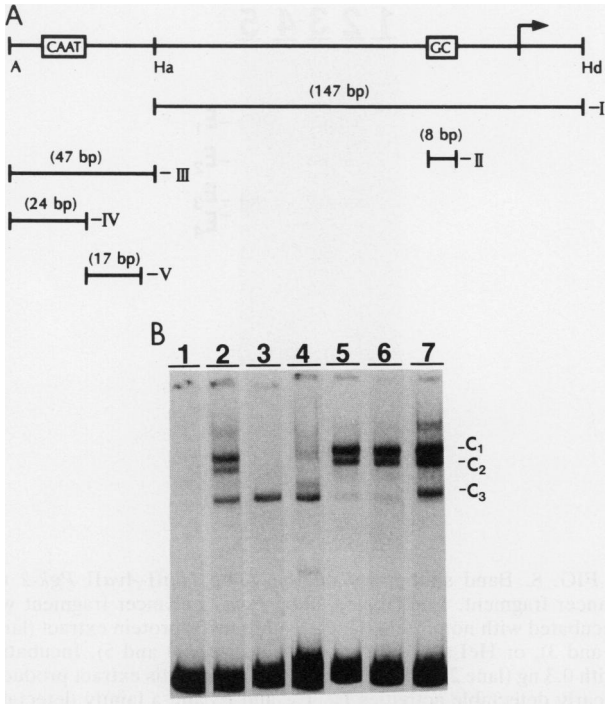

FIG. 4. (A) Schematic representation of the sizes and locations of unlabeled competitor fragments used to map binding activities in the *Pgk-2* core promoter. Oligonucleotide II was a 22-bp sequence containing 8 bp homologous to the *Pgk-2* GC box. Oligonucleotide IV was a 32-bp sequence containing 24 bp homologous to the *Pgk-2* CAAT-box region, beginning at 8 bp 5′ to the *Ava*II (A) site. Competitor fragment V was a concatemerized oligonucleotide representing a region adjacent to the CAAT box. Ha, *Hae*III; Hd, *Hin*dIII. (B) End-labeled *Pgk-2* core promoter fragment incubated with no protein (lane 1) or adult testis protein extract (lanes 2 to 8). In the absence of any competitor activities, C_1 to C_3 were seen (lane 2). A 50-fold excess of competitors I (lane 3) and II (lane 4) specifically competed for activities C_1 and C_2, while a 50-fold excess of competitor III (lane 5) or a 250-fold excess of concatemerized competitor IV (lane 6) specifically competed for activity C_3. Up to a 1,000-fold excess of competitor V failed to compete for any of these activities (lane 7).

either different forms or multimers of Sp1 present in the cell (6, 10).

(ii) The *Pgk-2* CAAT box binds purified CTF-1 protein. Antibodies to CTF-1 tend to cross-react with other members of the CTF family of transcription factors (34) and thus could not be used to unequivocally demonstrate CTF-1 binding to the *Pgk-2* CAAT box. We therefore obtained purified CTF-1 protein and incubated it with the labeled *Pgk-2* core promoter fragment for a band shift assay to define the specific electrophoretic mobility of the CTF-1–*Pgk-2* complex. This complex produced a band that was similar in electrophoretic mobility to band C_3 (Fig. 6, lane 3), strongly suggesting that band C_3 represents the interaction of CTF-1 with the *Pgk-2* CAAT box.

Analysis of regulatory sequences within the *Pgk-2* enhancer. To delineate protein-DNA interactions associated with the tissue-specific expression of *Pgk-2*, we focused on the 327-bp enhancer region which is located immediately upstream from the core promoter in the *Pgk-2* gene (Fig. 1) and which was shown to be necessary and sufficient to direct tissue-specific expression of reporter genes in transgenic mice (32). This fragment was too large to use intact for a

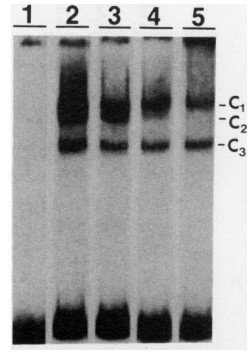

FIG. 5. Identification of Sp1-binding activities. Antibody to the transcription factor Sp1 was used to identify Sp1-binding activities in the *Pgk-2* core promoter. End-labeled *Pgk-2* core promoter fragment was incubated with no protein (lane 1) or adult testis protein extract (lanes 2 to 5). All three core promoter activities (C_1 to C_3) persisted after incubation with preimmune serum (2.0 μl [lane 2] or 0.2 μl [lane 3]). However, incubation with 0.2 μl (lane 4) or 2.0 μl (lane 5) of antibody to Sp1 resulted in a progressive diminution of activities C_1 and C_2 but not C_3, indicating that activities C_1 and C_2 represent binding of Sp1 to the *Pgk-2* promoter.

band shift assay and so was divided into two subfragments by endonuclease restriction at the *Hha*I site (Fig. 1). This produced a 157-bp *Pst*I-*Hha*I fragment (the 5′ half of the enhancer region) and a 170-bp *Hha*I-*Ava*II fragment (the 3′ half of the enhancer region) (Fig. 1), each of which was of a size that could be readily used in a band shift assay.

(i) Protein-DNA interactions in the 5′ half of the *Pgk-2* enhancer region. The labeled 157-bp *Pst*I-*Hha*I fragment was incubated with (i) nuclear extracts from adult mouse testicular cells, (ii) nuclear extracts from 5-day-old mouse testicular cells, and (iii) nuclear and whole cell extracts from

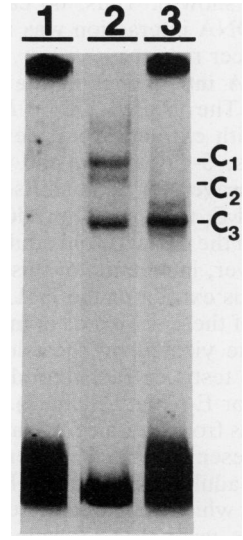

FIG. 6. Binding activities resulting from incubation of the end-labeled *Pgk-2* core promoter fragment with no protein (lane 1) or adult testis protein extract (lane 2) compared with those resulting from incubation of end-labeled *Pgk-2* core promoter fragment with purified CTF-1 protein (lane 3). Of three activities (C_1 to C_3) produced by incubation with adult testis extract, only one (C_3) was reproduced by incubation with purified CTF-1 protein, indicating that this activity specifically represents binding of CTF-1 to the *Pgk-2* core promoter.

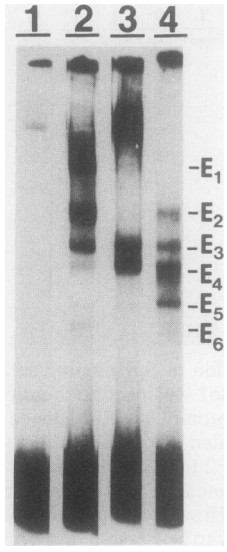

FIG. 7. Band shift assay on the 170-bp (*Hha*I-*Ava*II) enhancer region fragment. End-labeled 170-bp enhancer region fragment was incubated with no protein (lane 1) or adult testis protein extract (lane 2), 5-day testis protein extract (lane 3), or HeLa cell protein extract (lane 4). Six putative activities were detected (E_1 to E_6). Activities E_1 and E_2 were clearly detected in adult testis extracts but not in 5-day testis extracts and apparently not in HeLa cell extracts (a faint representation of E_2 is seen in lane 4, but this was not reproduced in subsequent experiments with smaller probe fragments; see Fig. 8). Activities E_3 to E_6 were detected in extracts from all three tissues, although activity E_4 was more abundant in 5-day testis and HeLa extracts than in adult testis extract.

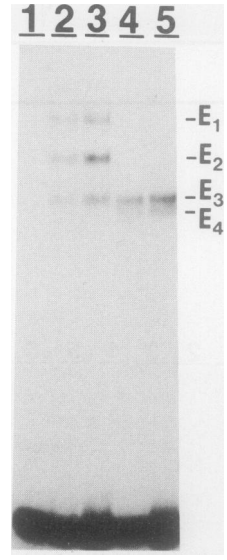

FIG. 8. Band shift assay on the 40-bp *Hin*fI-*Ava*II *Pgk-2* enhancer fragment. End-labeled 40-bp *Pgk-2* enhancer fragment was incubated with no protein (lane 1), adult testis protein extract (lanes 2 and 3), or HeLa cell protein extract (lanes 4 and 5). Incubation with 0.3 ng (lane 2) or 0.6 ng (lane 3) of adult testis extract produced clearly detectable activities E_1, E_2, and E_3 and a faintly detectable activity, E_4. Incubation with 0.3 ng (lane 4) or 0.6 ng (lane 5) of probe with HeLa extract produced activity E_3 and a relatively strong signal for activity E_4 but no representation of activity E_1 or E_2, indicating that activities E_1 and E_2 are specific to adult testis cells.

HeLa cells and then analyzed by band shift assay. In each case, three bands were consistently produced with similar electrophoretic mobilities regardless of the source of the extract (data not shown). Thus, no candidate for a tissue-specific protein-DNA interaction was identified in this portion of the enhancer region.

(ii) Protein-DNA interactions in the 3′ half of the *Pgk-2* enhancer region. The labeled 170-bp *Hha*I-*Ava*II fragment was incubated with extracts from the same three types of cells (adult testis, 5-day testis, and HeLa) as described previously and assayed for band shifts. Figure 7 shows that six bands (labeled E_1 to E_6) were produced by incubation of this fragment with the extract from adult testicular cells (Fig. 7, lane 2). However, incubation of this fragment with either the 5-day-old testis extract or the HeLa cell extracts reproduced only four of these six bands in intensities similar to or greater than those yielded by the adult testicular cell extracts. The 5-day testis extract reproduced bands E_3 to E_6 but not band E_1 or E_2 (Fig. 7, lane 3). In both nuclear and whole cell extracts from HeLa cells, band E_1 was absent and band E_2 was present at much lower intensity than that produced by the adult testis extract (Fig. 7, lane 4). Interestingly, band E_4, which appeared quite weak when the adult testis extract was used (Fig. 7, lane 2), showed a much higher intensity when either the 5-day testis or the HeLa extract was used (Fig. 7, lanes 3 and 4). In all cases, bands E_3, E_5, and E_6 were present at similar intensities regardless of the source of the extract and were thus not pursued as candidates for tissue-specific enhancer-binding activities. However, bands E_1, E_2, and E_4 did vary in appearance or intensity depending on the source of the extract, and thus these bands did represent candidates for tissue-specific protein-DNA interactions associated with the *Pgk-2* enhancer, which was thus delineated to a region no more than 170 bp in length (the *Hha*I-*Ava*II restriction fragment shown in Fig. 1).

Mapping of tissue-specific protein-DNA interactions in the *Pgk-2* enhancer. To map the location of the tissue-specific DNA-binding activities to specific subregions within the 170-bp *Pgk-2* enhancer, cold subfragment competitor experiments using the adult testis extract were performed. All three tissue-specific bands (E_1, E_2, and E_4), along with one non-tissue-specific band (E_3), mapped within the first 40 bp immediately upstream from the *Ava*II site (data not shown). To confirm that bands E_1 to E_4 all derive from binding activities to sequences that are completely within the 40-bp *Hin*fI-*Ava*II restriction subfragment, this fragment was labeled and used in a separate band shift assay (Fig. 8). Compared with the six bands produced by the 170-bp fragment incubated with adult testis extract (Fig. 7, lane 2), incubation with the 40-bp fragment reproduced only the four bands (E_1 to E_4) (Fig. 8, lanes 2 and 3) that had previously mapped to this region. When the 40-bp subfragment was incubated with HeLa cell extract, only bands E_3 and E_4 were reproduced (Fig. 8, lanes 4 and 5), but band E_4 was again much more prevalent than when the adult testis extract was used, while band E_3 showed a similar intensity in both cases. This result supports the previous conclusion that bands E_1, E_2, and E_4 represent tissue-specific binding activities. Note the absence of band E_2 when the 40-bp subfragment was incubated with HeLa cell extract, suggesting that the faint presence of this band when HeLa extracts were incubated with the 170-bp fragment may not have indicated a significant activity. These results thus delineate the tissue-specific,

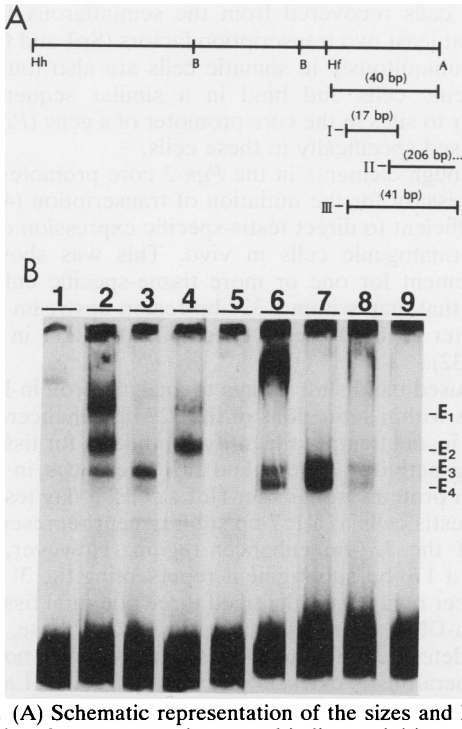

FIG. 9. (A) Schematic representation of the sizes and locations of competitor fragments used to map binding activities within the 40-bp *Hin*fI (Hf)-*Ava*II (A) *Pgk-2* enhancer fragment. (Note that subfragment II extends into the core promoter.) Hh, *Hha*I; B, *Bst*NI (B) End-labeled 170-bp *Hha*I-*Ava*II *Pgk-2* enhancer fragment was incubated with no protein (lane 1), adult testis protein extract (lanes 2 to 5), or 5-day testis protein extract (lanes 6 to 9). In the absence of any competitor, activities E_1 to E_4 were detected in adult testis extract (lane 2), and E_3 and E_4 were detected in 5-day testis extract (lane 6). Competitor I specifically competed for activities E_1 and E_2 in adult testis extract (lane 3) and failed to compete for any previously detected activity in 5-day testis extract (lane 7). Competitor II specifically competed for activities E_3 and E_4 in adult testis extract (lane 4) and in 5-day testis extract (lane 8). Competitor III competed for all four activities (E_1 to E_4) in adult testis (lane 5) and both activities (E_3 and E_4) in 5-day testis extract (lane 9). Note that the high-molecular-weight band that appears in lane 6 is not sequence specific, since it is competed for by both competitors I (lane 7) and II (lane 8).

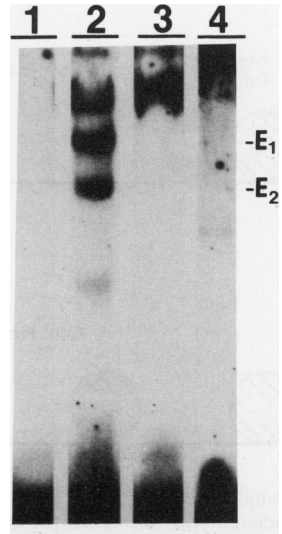

FIG. 10. Band shift assay on the 17-bp subfragment 3′ to the *Hin*fI site in the 40-bp *Pgk-2* enhancer region. End-labeled 17-bp *Pgk-2* enhancer subfragment was incubated with no protein (lane 1), adult testis protein extract (lane 2), 5-day testis protein extract (lane 3), or HeLa cell protein extract (lane 4). Binding activities E_1 and E_2 were detected in adult testis extract only, not in either 5-day testis extract or HeLa cell extract.

Pgk-2 enhancer activities to a region no more than 40 bp in length (the *Hin*fI-*Ava*II restriction fragment shown in Fig. 1).

To map the location of tissue-specific DNA-binding activities to specific sequences within the 40-bp *Hin*fI-*Ava*II *Pgk-2* enhancer region, cold competitor experiments using either adult or 5-day testis extracts (Fig. 9) were performed. A double-stranded oligonucleotide (Fig. 9A, oligonucleotide I), representing 17 bp from the 5′ half of the 40-bp *Pgk-2* enhancer, competed for both bands E_1 and E_2, but not band E_3 or E_4, when incubated with adult testis extract (Fig. 9B, lane 3). Similarly, oligonucleotide I failed to compete for bands E_3 and E_4 when incubated with the 5-day testis extract (Fig. 9B, lane 7). A competitor subfragment (Fig. 9A, subfragment II) that contained only the 3′-most 18 bp of *Pgk-2* sequence represented in the 40-bp enhancer region, plus additional *Pgk-2* sequence 3′ from the enhancer region, competed for only bands E_3 and E_4, not bands E_1 and E_2 (Fig. 9B, lanes 4 and 8). Finally, a 41-bp competitor subfragment (Fig. 9A, subfragment III), containing the 17 bp represented in oligonucleotide I plus an additional 20 bp of *Pgk-2* sequence 3′ to oligonucleotide I, competed for all four bands, E_1 to E_4 (Fig. 9B, lanes 5 and 9). These results indicate that the DNA-binding activities represented by bands E_1 and E_2 map to a 17-bp region in the 5′ half of the 40-bp *Pgk-2* enhancer, while activities represented by bands E_3 and E_4 map to an adjacent 18-bp region in the 3′ half of the 40-bp enhancer.

To confirm that the 17-bp region identified by the experiments in Fig. 9 includes all of the *Pgk-2* enhancer sequences required for DNA-binding activities E_1 and E_2 and that these activities are observed only in cells in which the *Pgk-2* gene is actively transcribed, oligonucleotide I (Fig. 9A) was labeled and incubated with adult testis extract, 5-day testis extract, and HeLa cell extract (Fig. 10). No bands equivalent to E_1 or E_2 were observed when either the 5-day testis or HeLa extracts were used (Fig. 10, lanes 3 and 4). However, both bands E_1 and E_2 were seen when the adult testis extract was used (Fig. 10, lane 2). This finding indicates that the positive portion (associated with expression) of the *Pgk-2* enhancer is delineated to the 17-bp sequence represented in oligonucleotide I.

DISCUSSION

The mammalian *Pgk-2* gene arose as a functional retroposon by reverse transcriptase-mediated processing of a transcript from the *Pgk-1* gene (29). Initial expression of this processed gene was apparently facilitated by coretroposition of the *Pgk-1* promoter sequence (27). Thus, the *Pgk-2* gene initially possessed a *Pgk-1*-like housekeeping promoter capable of directing only low-level, ubiquitous expression and has subsequently evolved a promoter sequence that directs high-level, tissue-specific expression (28). This process presumably involved the evolution of specific enhancer sequences in the *Pgk-2* promoter that bind tissue-specific transcription factors present in meiotic spermatogenic cells. In addition, minor sequence changes may have occurred in *Pgk-2* core promoter sequences to increase binding affinities

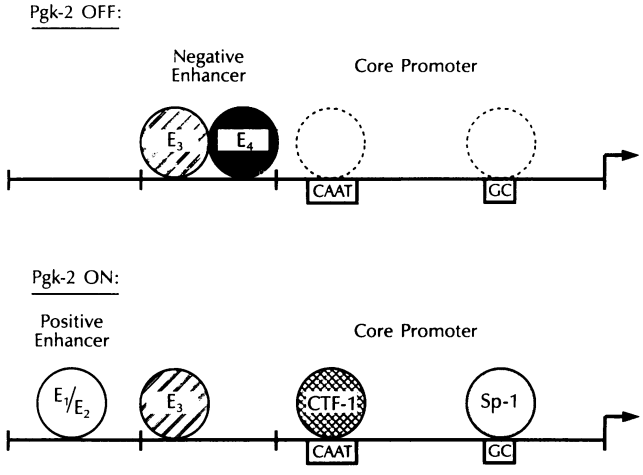

FIG. 11. Schematic representation of a model of protein-DNA interactions associated with the nonexpressed (*Pgk-2* off) and expressed (*Pgk-2* on) states of the *Pgk-2* gene. Binding of the ubiquitous E_3 activity is depicted associated with both states. The E_4 activity which is much more prevalent in nonexpressing tissues than in expressing tissues is shown specifically associated with the nonexpressed state of *Pgk-2*, not with the expressed state (see text for discussion). The E_1 and E_2 activities are shown specifically associated with the expressed state of *Pgk-2*. The ubiquitous transcription factors CTF-1 and Sp1 are shown bound to the CAAT and GC boxes, respectively, in the core promoter of an expressed *Pgk-2* gene. Whether or not these factors are bound to the promoter of the nonexpressed *Pgk-2* gene was not determined by this analysis.

for ubiquitous transcription factors, thus leading to an increased frequency of transcriptional initiation and a concordant increase in the level of expression of this gene (27). Two predictions of this scenario are that tissue-specific transcription factors should bind to sites within the *Pgk-2* enhancer region previously shown to be required to direct testis-specific expression of reporter genes in transgenic mice (32) and that ubiquitous transcription factors should bind in a sequence-specific manner to sites in the *Pgk-2* core promoter. Both of these predictions are validated by the results reported here.

Our band shift assays identified two types of binding activities of testicular cell nuclear proteins to the *Pgk-2* core promoter: (i) those that were specifically associated with the GC box and (ii) those that were specifically associated with the CAAT box. No other specific binding activities were observed in the core promoter. The binding activities associated with the *Pgk-2* GC box were shown by competition with antibodies to result from binding of the ubiquitous transcription factor Sp1 (Fig. 5). The ubiquitous transcription factor CTF-1 appears to be primarily responsible for the CAAT-binding activity in the *Pgk-2* promoter, as evidenced by the similar mobility of the complex produced when purified CTF-1 protein was incubated with the *Pgk-2* core promoter fragment (Fig. 6). Similar Sp1/GC-box- and CTF-1/CAAT-box-binding activities occurred when the *Pgk-2* core promoter fragment was incubated with protein extracts from any of three different tissues tested. This finding indicates that both Sp1 and CTF-1 are present and show similar sequence-specific DNA-binding capacities in both somatic and spermatogenic cells. It is unlikely that the binding activities observed with adult testis extracts were due solely to Sp1 and CTF-1 contributed by somatic cells in these preparations, since somatic cells make up less than 5% of the cells recovered from the seminiferous tubules (3). Thus, at least two transcription factors (Sp1 and CTF-1) that occur ubiquitously in somatic cells are also found in spermatogenic cells and bind in a similar sequence-specific manner to sites in the core promoter of a gene (*Pgk-2*) that is expressed specifically in these cells.

Although elements in the *Pgk-2* core promoter appear to be necessary for the initiation of transcription (4), they are not sufficient to direct testis-specific expression of this gene in spermatogenic cells in vivo. This was shown by the requirement for one or more tissue-specific enhancer elements that map within a 327-bp region upstream of the core promoter to direct testis-specific expression in transgenic mice (32).

We used band shift assays to analyze protein-DNA interactions within subregions of the 327-bp enhancer-containing region in an attempt to identify candidates for tissue-specific binding activities. We found no differences in binding of nuclear proteins from either HeLa cells, 5-day testis cells, or adult testis cells to a 157-bp subfragment representing the 5′ half of the 327-bp enhancer region. However, when we tested a 170-bp subfragment representing the 3′ half of the enhancer region, we identified three potential tissue-specific protein-DNA interactions (Fig. 7). Two of these, E_1 and E_2, were detected only in adult testis extracts, not in either prepuberal testis extracts or extracts from HeLa cells. The third activity, E_4, was detected in abundance in extracts from prepuberal testis or HeLa cells but only at a very low level in adult testis extracts. A fourth activity, E_3, was detected at similar levels in extracts from all three tissue sources. Thus, activities E_1 and E_2 appear to be specifically associated with the expressed state of *Pgk-2*, whereas activity E_4 may be strictly, or at least predominantly, associated with the nonexpressed state (see below). Activity E_3 appears to be equally prevalent in both the expressed and nonexpressed states.

More precise competition experiments showed that all three tissue-specific binding activities, E_1, E_2 and E_4, along with the ubiquitous E_3 activity, map to a region within the first 40 bp immediately upstream from the core promoter. Using specific oligonucleotides, we then showed that activities E_1 and E_2 map within a 17-bp region (-142 to -125) in the 5′ half of this 40-bp fragment, while activities E_3 and E_4 map to an immediately adjacent (and slightly overlapping) 18-bp region (-127 to -110) in the 3′ half of the 40-bp fragment.

Activities E_1 and E_2 could not be distinguished on the basis of either specific binding sites or tissue specificity. Thus, they could represent two forms of a single protein-DNA interaction (e.g., multimers), or they could be distinct activities. E_1 and E_2 were distinguished from E_3 and E_4 on the basis of both binding sites and tissue specificity. No differences could be resolved in binding sites for E_3 and E_4 even though these activities showed differences in tissue specificity. The latter finding suggests E_3 and E_4 are separate activities that bind adjacent to each other; however, in the absence of mapping or other data distinguishing these binding sites, we cannot rule out the possibility that E_3 and E_4 also represent alternate forms of a single activity.

The testis contains many different somatic and germ cell types. In the adult testis, *Pgk-2*-expressing spermatids and spermatocytes make up a large majority of the cells (3). At 5 days of age in the mouse, the prepuberal testis lacks both of these cell types and is composed primarily of interstitial cells, Sertoli cells, and spermatogonia, none of which express *Pgk-2*. The abundant representation of binding activity

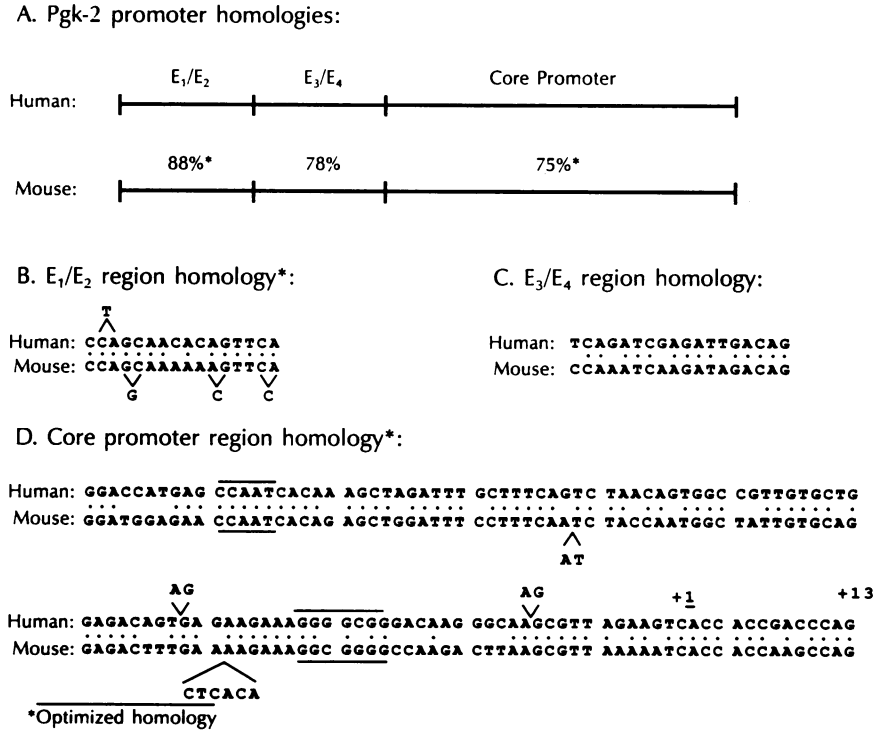

FIG. 12. (A) Homologies and relative locations of equivalent regions of the human and mouse *Pgk-2* promoters. A similar arrangement of putative positive (E_1/E_2) and negative (E_4) enhancer elements immediately 5′ to the core promoter region is evident in each case. (B) Optimal alignment of the E_1/E_2-binding region from the human *Pgk-2* promoter with the equivalent region in the mouse *Pgk-2* promoter. A match of 14 of 17 bp is achieved in this manner. (C) Alignment of the E_3/E_4-binding region from the human *Pgk-2* promoter with the equivalent region in the mouse *Pgk-2* promoter. A match of 15 of 18 bp is achieved. (D) Optimal alignment of the core promoter regions of the human and mouse *Pgk-2* promoters. An overall homology of 75% is achieved. Note the conservation and similar positions of the CAAT- and GC-box sequences (indicated by horizontal lines) in each promoter.

E_4 in extracts from 5-day testis compared with the significantly reduced representation of this activity in extracts from adult testis (Fig. 7) suggests that the protein responsible for this activity may be absent from spermatocytes and spermatids and is therefore underrepresented in extracts from adult testis. The occurrence of this same activity in HeLa cell extracts further suggests that this protein-DNA interaction is specifically associated with a repressed state in the *Pgk-2* promoter and that E_4 may represent a negative regulator of *Pgk-2* transcription. It is possible that all of the E_4 activity in prepuberal testis extracts is contributed by somatic cells and that an absence of E_4 in spermatogonia is a prerequisite to subsequent initiation of *Pgk-2* transcription in spermatocytes. Further transcriptional studies will be required to test these possibilities.

The unique representation of E_1- and E_2-binding activities in adult testis extracts suggests these activities are contributed by proteins specific to spermatocytes and spermatids and are thus integral members of the active transcription complex associated with the *Pgk-2* promoter in these cells. The apparent absence of these proteins in spermatogonia, as evidenced by the absence of these binding activities in 5-day testis extracts, suggests that subsequent expression of these proteins in spermatocytes may be a final positive signal required for activation of *Pgk-2* transcription.

The tissue and cell type specificities of activities E_1, E_2, and E_4 discussed above suggest that differential expression of these DNA-binding proteins forms a one- or two-step switch required to activate *Pgk-2* transcription. One possible model of the *Pgk-2* enhancer switch mechanism is schematically represented in Fig. 11. The fully repressed state in the *Pgk-2* promoter occurs in somatic cells and is characterized by binding of a negative regulator, E_4, possibly in conjunction with a ubiquitous regulator, E_3. Presumably the ubiquitous factors CTF-1 and Sp1 are not bound to the core promoter of the repressed *Pgk-2* gene; however, our data do not directly address this question. The fully activated state occurs in spermatocytes and spermatids and requires the action of one or more positive regulators, E_1 and E_2, in the absence of the negative regulator E_4. Whether this transition occurs as a single step, with cessation of expression of the E_4 protein being contemporaneous with the initiation of expression of the E_1/E_2 protein(s) in spermatocytes, or as a two-step process in which cessation of activity E_4 precedes initial expression of E_1 and E_2, is yet to be determined. A third, and perhaps most attractive, alternative is that activity E_4 may be unique to somatic cells and may never occur in any spermatogenic cell type. Thus, the spermatogenic cell lineage would be predisposed to express *Pgk-2*, by virtue of the absence of E_4, as soon as activities E_1 and E_2 are expressed in spermatocytes. Further analyses by band shift assays using extracts from purified populations of spermatogenic cell types or in vivo footprinting of protein-DNA interactions in these cells should help to resolve these possibilities. Final confirmation of the significance of these proposed enhancer sequences in appropriate transcriptional assays (e.g., transgenic mice) will also be required to substantiate this model.

The fact that the human *Pgk-2* promoter directed appropriate spermatogenic cell-specific expression of reporter

genes in transgenic mice (32) suggests that similar enhancer elements are present in the human and mouse *Pgk-2* promoters and that any candidate sequences for these elements should be conserved in these two species. Figure 12 shows that the sequences that we have identified as putative binding sites for activities E_1 to E_4 satisfy this criterion. Thus, on the basis of sequence similarities, we have identified homologous regions in similar locations relative to the core promoter that could act as binding sites for E_1 and E_2 and for E_3 and E_4 (Fig. 12A). Using maximized alignment of the sequences in these regions, we found that the region responsible for activities E_1 and E_2 in the human *Pgk-2* promoter is 88% homologous to the similar region in the mouse *Pgk-2* promoter (Fig. 12B). Although this homology is achieved only when one base from the human sequence and three bases from the mouse sequence are looped out, this region from the mouse *Pgk-2* promoter remains the best candidate for the positive enhancer sequence, since (i) it is in a similar position relative to the putative E_3/E_4-binding region and core promoter region in the mouse *Pgk-2* promoter and (ii) it shows more similarity to the human E_1/E_2 region than does any other 17- to 20-bp sequence in the first 400 bp upstream from the transcriptional start site in the mouse *Pgk-2* gene. The region binding E_3 and E_4 in the human *Pgk-2* gene shows 78% homology with the analogous region in the mouse gene (Fig. 12C), and the two *Pgk-2* core promoters are 75% homologous (Fig. 12D).

Pgk-2 is representative of a battery of genes, the expression of which is initiated during meiotic prophase in spermatogenic cells (37). This suggests the possibility of coordinate regulation and thus common mechanisms governing the onset of transcription of these genes. It will be of interest to determine whether other genes initially expressed at the onset of meiosis during spermatogenesis in mammals share enhancer sequence motifs similar to those that we have described here for the *Pgk-2* gene. In addition, it will be of interest, and necessary, to carry out transcription analyses (e.g., with transgenic mice) to determine which of these enhancer sequences are absolutely required and in vivo footprinting analysis to determine which protein-DNA interactions actually occur in spermatogenic cells and in what order both enhancer DNA-binding activities and core promoter-binding activities are established during spermatogenesis in the mouse.

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