Differential Appearance of DNase I-hypersensitive Sites Correlates with Differential Transcription of Pgk Genes during Spermatogenesis in the Mouse*

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Two functional genes encoding phosphoglycerate kinase (PGK) are differentially expressed during spermatogenesis in the mouse. Expression of the X-linked Pgk-1 gene is repressed coincident with X chromosome inactivation during prophase of meiosis I. At this same stage, expression of the autosomal Pgk-2 gene is initiated by tissue-specific mechanisms. To investigate the role of chromatin structure in these processes, we have examined the appearance and disappearance of DNase I-hypersensitive (DH) sites in each gene, and correlated this with transcriptional activity as measured by nuclear run-off analysis at specific stages of spermatogenesis. Our results demonstrate that the occurrence of DH sites is correlated to periods of active transcription. Results with the Pgk-2 gene indicate that transcriptional inactivation of the X chromosome in spermatogenic cells may not be as complete as that in somatic cells, and that maximum repression may be limited to a very transient period during the pachytene stage of first meiotic prophase. Results with the Pgk-2 gene indicate that DH sites appear coincident with, or just prior to, transcriptional activation of this gene. The implications of these results are discussed with respect to the role of X chromosome inactivation in spermatogenic cells and the developmental order of molecular events that regulate differential gene expression during spermatogenesis.

Phosphoglycerate kinase (PGK) is a key enzyme involved in the metabolism of glucose or fructose during glycolysis. In mammals, PGK is encoded by two functional genes, Pgk-1 and Pgk-2 (1). Pgk-1 is an X-linked gene, and is ubiquitously expressed at relatively low levels in all somatic cells. The Pgk-2 gene is autosomal and is expressed in a tissue-and cell-type specific manner at relatively high levels during spermatogenesis, specifically in meiotic spermatocytes and postmeiotic round spermatids (1, 2). In mammalian spermatogenic cells, transcriptional repression of the Pgk-1 gene occurs as a result of X chromosome inactivation (XCI) during prophase of meiosis I (2, 3). At the same time, the tissue-specific expression of the autosomal Pgk-2 gene is initiated (2, 3).

Although the Pgk-1 gene is expressed ubiquitously in somatic cells, tissue-specific differences in levels of Pgk-1 transcript have been noted (4), suggesting the Pgk-1 promoter confers some degree of transcriptional regulation on the active gene. Dramatic differences have been reported for regulatory parameters associated with the Pgk-1 promoter on the transcriptionally active and inactive X chromosomes in mammalian female somatic cells. These include hypomethylation of specific CpG sites (5, 6), DNase I-hypersensitive sites (7, 8), and binding transcription factors (5, 9), all of which are associated with the constitutively expressed Pgk-1 gene on the active X, but are absent from the terminally repressed Pgk-1 gene on the inactive X chromosome. However, some of these differences do not distinguish the active and inactive X chromosomes in germ cells. For instance, it has been shown that the inactivated X chromosome in germ cells lacks the repressive hypermethylation associated with the inactivated X chromosome in somatic cells (2, 10, 11). This is probably because unlike the situation in female somatic cells, inactivation of the X chromosome in germ cells is a transient event (reviewed in Ref. 12).

Differences in the mechanism of XCI in germ cells and somatic cells could reflect a different function provided by this phenomenon in each cell type. In somatic cells, XCI achieves dosage compensation between males and females with respect to expression of X-linked genes. However, in germ cells the dose of X-linked gene expression differs markedly between spermatocytes which possess zero active X chromosomes, and oocytes, in which both X chromosomes are active (reviewed in Ref. 12). Thus, the function of differential X chromosome activity during gametogenesis in males and females is not dosage compensation.

The tissue-specific occurrence of Pgk-2 mRNA in the testis implies stringent transcriptional control conferred by the Pgk-2 gene promoter. This promoter consists of a core region, defined on the basis of its ability to direct transcription of a reporter gene in a heterologous cell culture system (13), plus an upstream enhancer region which is required to direct appropriate tissue- and cell-type specific expression of a reporter gene in transgenic mice (14), and in which binding sites reside for testis-specific nuclear factors (15). Transcription and protein-binding assays demonstrate the critical role that transcription factors play in regulating gene expression. However, the ability of transcription factors to exert such regulation is dependent upon access of these factors to their cognate binding sites in each gene promoter. This access is associated with changes in the structure of the chromatin in which these sites are embedded. Thus, the presence of an "open" accessible chromatin structure represents a critical corollary to the state of active transcription.

Toward the goal of understanding the molecular mechanisms underlying the repression of Pgk-1 transcription and the...
initiation of Pgk-2 transcription during spermatogenesis, we have examined both the occurrence and the relative timing of changes in chromatin structure in relation to transcription factor binding and the actual initiation or cessation of transcription of these genes in specific spermatogenic cell types. Our results suggest that the appearance of DH sites may occur coincident with, or just prior to transcription factor binding and initiation of transcription of the Pgk-2 gene. Our results also indicate that maximum transcriptional repression during spermatogenesis of X-linked genes such as Pgk-1 may be less complete, and more transient than previously thought.

EXPERIMENTAL PROCEDURES

Isolation of Nuclei from Liver and Germ Cells—Nuclei from liver tissue were isolated by Dounce homogenization in the presence of 0.5% Triton X-100 as described elsewhere (16). Spermatogenic cells from CD-1 mice (Charles River) were separated by unit gravity sedimentation (Sta Put gradient) (17, 18). Primary type A and a mixture of type A and type B spermatogonia were isolated from testes of prepuberal mice at 15 min of age, respectively. Somatic Sertoli cells were also isolated from mice at these ages. Preleptotene, leptotene/zygotene, and early pachytene spermatocytes were recovered from testes of 18-day-old mice. Testes from adult mice were used as the source of late pachytene spermatocytes, round spermatids and testicular spermatozoa. The latter were prepared as sonication-resistant cells as described previously (2).

Plasma membranes of spermatogenic and Sertoli cells were disrupted by resuspension in 0.25% Triton X-100 followed by pipetting with a plastic Pasteur pipette. Liberated nuclei were recovered by centrifugation at 4 °C for 7 min at approximately 2000 × g through a 0.8 M sucrose solution in isosmotic buffer (25 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.15 mM spermine, 1.5 mM spermidine, 50 mM Tris-hydrochloride (pH 7.0)) as described elsewhere (16).

DNase I Treatment—Nuclei at a concentration of 2 × 10^9/ml from liver cells, Sertoli cells, or spermatogenic cells were resuspended in DNase I digestion buffer (50 mM KCl, 15 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 0.25 M sucrose, 3 mM MgCl_2, 0.05 mM CaCl_2) (19). Aliquots (0.1–0.5 ml) were digested with increasing concentrations of DNase I (0–30 μg/ml) (Worthington) at 37 °C for 7 min. Each reaction was stopped by addition of SDs to 0.5% and EDTA to 10 mM. Genomic DNA was purified by digestion with 50 μg/ml proteinase K overnight at 37 °C, followed by organic extraction and precipitation in two volumes of ethanol. Purified DNA was resuspended at a concentration of 1 mg/ml in TE buffer (10 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.0)).

Southern Analysis—Aliquots of 15 μg of purified DNA each were digested to completion using 50–55 units Apal (Life Technologies, Inc.) at 30 °C for Pgk-1, or 30–40 units of EcoRI (Promega) at 37 °C for Pgk-2, each supplemented with 50–60 units of RNase A, for 16 h. The digested DNA was precipitated in ethanol and then resuspended in 20 μl of TE buffer. Samples of 7–10 μg were electrophoresed through 0.8% agarose and transferred to Gene Screen Plus membrane (DuPont) according to the manufacturer's instructions. After baking for 2 h at 80 °C under vacuum, membranes were hybridized overnight with a DNA probe labeled by random priming to a specific activity of ~1 × 10^9 cpm/μg with [α-32P]dCTP (Megaprime DNA labeling systems, Amersham Corp.). Membranes were washed at 55 °C (for the PGk-1 gene) or 65 °C (for the PGk-2 gene) in 0.2 × SSC (1 × SSC = 0.15 M NaCl plus 0.1 M sodium citrate) and 0.1% SDS, then exposed to x-ray film with intensifying screens.

The Pgk-1 gene was analyzed with a 1.05-kb Xhol-Apal probe fragment from the first intron of the mouse Pgk-1 gene (20). The Pgk-2 gene was analyzed with a 1.6-kb EcoRI-HindII probe fragment from the 5'-flanking region of the mouse Pgk-2 gene (21). These probe fragments were isolated by agarose gel electrophoresis and recovered either by digestion with base agarase (New England Biolabs) or electroelution (18) of isolated bands according to the manufacturer's instructions. The location of these probe fragments in each respective gene is schematically represented in Fig. 1.

Bal-31 Exonuclease Assay—Nuclei isolated as described above were suspended in 90 ml Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 10 mM MgCl_2, 0.1 mM EGTA, 0.3 mM sucrose and 0.1 mM phenylmethylsulfonyl fluoride at a concentration equivalent to 0.5 mg of nucleic acid in 2 ml and incubated for 30 min with 1000 units/ml Apal at 37 °C for Pgk-1 or a similar concentration of EcoRI at 37 °C for Pgk-2. Partially digested nuclei were then recovered by centrifugation at 10,000 × g for 1 min. The nuclear pellet was resuspended in 2 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 12 mM MgCl_2, 12 mM CaCl_2, 1 mM EDTA, 0.3 M sucrose, and 0.1 mM phenylmethylsulfonyl fluoride. Aliquots (0.5 ml each) were digested with increasing concentrations of Bal-31 exonuclease (2.5, 5.0, and 7.5 units/ml). Each reaction was stopped by the addition of SDS to 0.5% and EDTA to 20 mM. DNA was purified as described previously (22). Membrane filters were prepared as sonication-resistant cells as described previously (2).

Localization of DH and Bal-31-Protected Sites—Mapping of the hypersensitive sites in each Pgk gene was accomplished by comparing the size of minor bands indicative of the presence of one or more DH sites with that of parent fragments produced by digestion with specific restriction endonucleases as shown in Fig. 1. For the Pgk-1 gene, a 6.6-kb parent fragment representing the 5' portion of the gene was produced by digestion with Apal, and hybridization with probe A1, which abuts the 3' end of this fragment. Interestingly, a smaller parent band of about 5.9 kb was also produced by Apal digestion (Figs. 2 and 4). This could be due to a polymorphism for Apal restriction sites in this gene; however, it is clearly not a product of DNase I digestion and therefore does not impact on the analysis of DH sites in this gene. Similarly, for the Pgk-2 gene, genomic DNA was digested with EcoRI to yield a 5.1-kb parent fragment from the 5' portion of the gene, which was then hybridized with probe A2, which abuts the 5' end of this fragment. A similar approach was used to map Bal-31 sites. The location of protected sites was determined by Southern analysis (Fig. 1). For the Bal-31 sites at which digestion by Bal-31 exonuclease was blocked by bound protein was determined by analyzing the same parent fragments with the same corresponding probes as described for the DH sites above. Bands of sizes smaller than the parent fragment were indicative of a protected site.

For both DH and Bal-31 sites, minor bands were sized by electrophoresis by comparison with markers of known sizes. The thickness of each band representing a DH site provided a rough estimate of the extent of each site. Because of the variability in the thickness of these bands, we analyzed the thickest band to provide an indication of the maximum extent of each site, which is shown in Fig. 3. Because of the location of each probe, our Bal-31 analysis detected the 5' boundary of each protein-binding region in the PGk-1 gene, and the 3' boundary of each protein-binding region in the PGk-2 gene (Fig. 3). As expected, bands of sizes smaller than the parent fragment were indicative of a protected site.
Fig. 2. DNase I-hypersensitive and Bal-31-protected sites in the 5′-flanking region of Pgk genes in expressing cell types.

Liver cells and total testicular cells were used for analysis of expressed Pgk-1 and Pgk-2 genes, respectively. Isolated nuclei were left undigested (0 lanes) or digested with increasing concentrations of DNase I or Bal-31 (open triangle above each blot), then further digested with Apal (Pgk-1) or EcoRI (Pgk-2) and analyzed for DH sites by Southern blotting. Two DNase I/Bal-31 sites (sites 1 and 2) were detected for the expressed Pgk-1 gene in liver cells, and one DNase I site (site 1) and two Bal-31 sites (sites 1 and 3) were detected in the expressed Pgk-2 gene in total testis cells. Two additional DNase I sites (sites 2 and 3) were detected in the expressed Pgk-2 gene in purified populations of certain spermatogenic cell types, but none of these sites was found in nonexpressing cell types (see Fig. 5).

relative to the transcription start point (+1) (Fig. 3).

Nuclear Run-on Assays—Nuclei from isolated populations of dispersed liver cells, adult pachytene spermatocytes and round spermatids were prepared as described above, resuspended in glycerol storage buffer [50 mM Tris-HCl (pH 8.3), 40% (v/v) glycerol, 5 mM MgCl2, 5 mM dithiothreitol, and 0.1 mM EDTA], and stored at −70 °C until use. Nuclei were thawed and incubated at 30 °C for 30 min to reinitiate transcription in the presence of [α-33P]UTP (specific activity, 3,000 C/mM) (DuPont) as described (22, 23). Newly synthesized RNA was isolated using a micro RNA isolation kit (Stratagene) according to manufacturer’s instructions. 15 × 106 cpm of labeled RNA were added to 2–3 ml of hybridization buffer (16 mM TES (pH 7.4), 1% SDS, 10 mM EDTA, 250 μg/ml E. coli RNA, 0.3 mM NaCl, 1 × Denhardt’s buffer (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 0.25% powdered milk) and used to probe dot blots carrying mouse Pgk-1 and Pgk-2 sequences spotted onto Gene Screen membranes (DuPont) in quantities of 10 ng, 100 ng, and 1 μg. The Pgk-1 sequence was a 900-bp BgII/XbaI fragment, and the Pgk-2 sequence was a 900-bp AclI fragment, each representing coding sequence derived from the respective mouse cDNA clones (24, 25). SP6 plasmid sequence was used as a negative control (data not shown). Hybridizations were carried out for 48 h at 65 °C, followed by washing with 2 × SSC, 0.1% SDS at room temperature 2 × 15 min and with 0.2 × SSC, 0.1% SDS at 65 °C for 10 min, and analysis of signal using a Molecular Dynamics PhosphorImager.

RESULTS

Expression-specific DNase I-hypersensitive and Bal-31 Exonuclease-protected Sites in the 5′-flanking Regions of the Pgk Genes—Gene promoter sequences that act as binding sites for factors involved in the regulation of transcription are usually located within nucleosome-free regions of chromatin (26). The absence of nucleosomes results in these regions becoming hypersensitive to digestion with endonucleases such as DNase I; however, the presence of bound regulatory proteins protects these same regions from digestion with exonucleases such as Bal-31 (reviewed in Ref. 26).

To identify such regions in the murine Pgk genes, we first investigated the presence of DH sites in the 5′-flanking region, exon 1, and part of intron I of the Pgk-1 gene, and in the 5′-flanking region, coding region, and 3′-flanking region of the intronless Pgk-2 gene, in tissues known to express each gene. Male liver was used as an example of a tissue known to express the Pgk-1 gene. The results illustrated in Fig. 2 show that limited digestion of nuclei with DNase I generated two minor bands in addition to the 6.6-kb parent band in the expressed Pgk-1 gene. One minor band of 2.1 kb (Pgk-1 DH site 1) appeared after digestion with 5 μg/ml DNase I, while a second minor band of 1.3 kb (Pgk-1 DH site 2) appeared after digestion with 10 μg/ml DNase I. Hypersensitive sites were detected in the expressed Pgk-2 gene in nuclei from adult testis tissue (Fig. 2). In addition to the 5.1-kb parent fragment, one minor band of 2.7 kb (Pgk-2 DH site 1) was produced by digestion with 5 μg/ml DNase I. In subsequent experiments, two additional sites (Pgk-2 DH sites 2 and 3) were seen in spermatocytes and spermatids after digestion with 0.625 μg/ml DNase I (Fig. 5).

To determine if these DH sites are unique to the expressed state of each Pgk gene, we investigated nonexpressing tissues. Only faint DH sites were detected in the Pgk-1 gene in pachytene spermatocytes in which the X chromosome is transcriptionally repressed (Fig. 4). Similarly no DH sites were detected in the Pgk-2 gene in any nonexpressing somatic cell type, including liver and Sertoli cells (Fig. 5).

We used the Bal-31 exonuclease protection assay to confirm that the expression-specific DH sites we detected correspond to sites of bound protein factors in each Pgk gene. Specific protection sites were detected in the Pgk-1 gene in expressing liver nuclei (Fig. 2), and two sites of protection were also detected in the Pgk-2 gene in expressing testis cell nuclei (Fig. 2).

Mapping of DNase I and Bal-31 Sites in Expressed Pgk Genes—For the Pgk-1 gene, DH site 1 mapped to a region from +14 to −331 in the 5′-flanking region (Fig. 3), while DH site 2 mapped to +606 to +706 in intron I. Exonuclease Bal-31 blocks mapped to sites corresponding to both of these DH sites. The 5′ boundary of the first block was observed at −484 (Fig. 3) and that of the second block was seen at +526.

For the Pgk-2 gene, DH site 1 mapped to a region from −199 to +21 in the 5′-flanking region (Fig. 3), while DH site 2 mapped to a region in the coding sequence at +300 to +322, and DH site 3 mapped to the 3′-untranslated region at +1399 to +1649. Exonuclease Bal-31 blocks mapped within Pgk-2 DH sites 1 and 3. The 3′ boundary of the first block was observed at +1 (Fig. 3) and the second block was seen at +1378.

Analysis of Chromatin Structure of the Pgk-1 Gene during Spermatogenesis—The X-linked Pgk-1 gene becomes transcriptionally repressed during meiotic prophase in male germ cells. We assayed the presence or absence of the two expression-specific DH sites (1 and 2) in this gene in spermatogenic cells at premeiotic, meiotic, and postmeiotic stages. Nuclei from premeiotic primitive type A, and a mixture of types B and C spermatogonia, and from meiotic preleptotene, leptotene plus zygotene, early pachyteny, and adult pachytene spermatocytes, and from postmeiotic round spermatids and testicular spermatozoa were each analyzed (Fig. 4). A strong representation of Pgk-1 DH site 1 was detected in liver cells after digestion with 5 μg/ml DNAse I/ml of nuclear suspension. This same site was detected at varying intensities after digestion with a similar concentration of DNAse I in Sertoli cells, primitive type A spermatogonia, types B + C spermatogonia, leptotene + zygotene spermatocytes, after digestion with 7.5 μg/ml DNAse I in round spermatids, and with 10 μg/ml DNAse I in preleptotene spermatocytes. Unlike either the other primary spermatocyte cell types or the round spermatids, pachytene spermatocytes (from either prepuberal or adult males) showed only a faint DH site by autoradiography, even after digestion with 10 μg/ml DNAse I. Nevertheless, this weak site was clearly detectable when analyzed on a PhosphorImager (Fig. 4).

Pgk-1 DH site 2 was detected in liver nuclei after digestion with 10 μg/ml DNAse I. This site was not detected in any of the spermatogenic cell types we analyzed except for a faint representation in nuclei from round spermatids after digestion of nuclei with 15 μg/ml DNAse I. Neither site 1 nor site 2 were
detected in the Pgk-1 gene in testicular spermatozoa at any concentration of DNase I used.

Analysis of Chromatin Structure of the Pgk-2 Gene during Spermatogenesis—Transcription of the autosomal Pgk-2 gene is initiated at the onset of meiosis in primary spermatocytes (2). We analyzed the appearance and disappearance of DH sites.
in the Pgk-2 gene using the same male germ cell types as described above for the Pgk-1 gene. As noted above, one DH site was detected in the Pgk-2 gene in a mixed population of cells from the adult testis. Surprisingly, in addition to this one site, two other sites were detected in enriched populations of specific meiotic or postmeiotic spermatogenic cell types, including preleptotene, a mixture of leptotene and zygotene, early pachytene, and late pachytene spermatocytes, round spermatids, and testicular spermatozoa. Isolated nuclei were left undigested (0 lanes) or digested with increasing concentrations of DNase I (open triangle above each blot) and analyzed for DH sites. Three sites (Pgk-2 DH sites 1–3) were detected in all spermatocytes and and spermatids, but not in somatic liver or Sertoli cells, nor in primitive type A spermatogonia or testicular spermatozoa. Faint detection of these sites in type A + B spermatogonia was possible when a PhosphorImager was used (inset at bottom).

FIG. 5. DNase I-hypersensitive sites in the Pgk-2 gene during spermatogenesis. DH sites in the 5′-flanking region, coding region and the 3′-flanking region of the Pgk-2 gene in male somatic cells (liver cells and testicular Sertoli cells), and spermatogenic cells (primitive type A spermatogonia (prim. A gonia), a mixture of type A + B spermatogonia (A + B gonia), preleptotene, leptotene + zygotene, prepuberal (early) pachytene, and adult pachytene spermatocytes, round spermatids, and testicular spermatozoa. Isolated nuclei were left undigested (0 lanes) or digested with increasing concentrations of DNase I (open triangle above each blot) and analyzed for DH sites. Three sites (Pgk-2 DH sites 1–3) were detected in all spermatocytes and and spermatids, but not in somatic liver or Sertoli cells, nor in primitive type A spermatogonia or testicular spermatozoa. Faint detection of these sites in type A + B spermatogonia was possible when a PhosphorImager was used (inset at bottom).

Analysis of Ongoing Transcription of the Pgk Genes at Meiotic and Postmeiotic Stages of Spermatogenesis—Because of the reappearance of Pgk-1 DH site 1, and the increased intensity of Pgk-2 DH site 1 in nuclei from round spermatids relative to those from pachytene spermatocytes, we used the nuclear runoff assay to examine the status of ongoing transcription of each Pgk gene in these two cell types, as compared to that in somatic liver cells (Fig. 6). Our results indicate that ongoing transcription of the Pgk-1 gene occurs at a maximum level in somatic cells, and is minimal in pachytene spermatocytes. Interestingly, despite the significant reappearance of Pgk-1 DH site 1 in round spermatids, we observed only a slight increase in the level of new transcription of this gene in these cells relative to that in somatic liver cells (Fig. 6).

In contrast to the Pgk-1 gene, the Pgk-2 gene showed no ongoing transcription in somatic liver cells, but a moderate level of transcription in pachytene spermatocytes, and a maximal level of transcription in postmeiotic round spermatids (Fig. 6).

DISCUSSION

DH sites found within 1 kb of the 5′-flanking end of an active gene are typically most relevant to the control of gene expression (27), mapping to core promoters (16, 28–30), enhancers (30), and/or silencers of transcription (31–33) (reviewed in Ref. 26). Of the two DH sites we observed in the active Pgk-1 gene, one (DH site 1) mapped to a 5′-promoter/enhancer region previously defined in transient transfection assays (34) (Fig. 3). A second site (DH site 2) detected in some expressing cell types
mapped to the first intron of the Pgk-1 gene. That these hypersensitive sites correspond to protein-binding sites was further indicated by resistance to exonuclease Bal-31 digestion that mapped to similar locations. While detection of DH sites in intronic sequences is not unprecedented (33, 35–37), the potential significance of this region for regulation of the Pgk-1 gene expression is unclear. McBurney et al. (38) found that a fragment containing the mouse Pgk-1 promoter/enhancer sequences plus the first exon and part of the first intron gave maximal expression of a reporter gene in transient transfection assays. This fragment spans a region containing both of the DH sites we observed in the expressed Pgk-1 gene in male liver cells. However, they also demonstrated that a fragment lacking the intronic sequences (and hence DH site 2) could still direct transcription, albeit at a slightly reduced level (38). The apparent absence of Pgk-1 DH site 2 in the expressed Pgk-1 gene in either premeiotic spermatogonia or somatic Sertoli cells suggests it is not critical for expression of this gene, at least in testicular cells.

Of the three DH sites we observed in the active Pgk-2 gene, only one (DH site 1) maps to the promoter/enhancer region (Fig. 3), while the other two map to regions within, and immediately 3’ to, the coding sequence. Previous studies with transgenic mice demonstrated that the Pgk-2 promoter plus enhancer region alone are sufficient to direct appropriate tissue-specific expression of a reporter gene (14). Thus, we assume that Pgk-2 DH site 1 is the only one associated with a site that is critical for tissue-specific regulation. Previous gel-shift experiments indicated potential tissue-specific enhancer and repressor activities in this same region (15). In this respect it is noteworthy that we observed no DH sites in nonexpressing cell types including liver and Sertoli cells (Fig. 5). This indicates there is probably not constitutive binding of a repressor protein to this region of the Pgk-2 promoter in nonexpressing cells in vivo.

FIG. 6. Nuclear run-off transcription assay for ongoing Pgk gene transcription during spermatogenesis. Nuclei from purified populations of male liver cells, adult pachytene spermatocytes, and round spermatids were used to generate [32P]UTP-labeled run-off transcripts. The labeled RNA was hybridized to dot blots carrying 10-ng, 100-ng, and 1-μg quantities of a 900-bp denatured fragment of the mouse Pgk-1 coding sequence or the mouse Pgk-2 coding sequence. Relative active transcription of the Pgk-1 gene is indicated in somatic liver cells, while only a very faint signal is detected in pachytene spermatocytes, and a slightly stronger signal is seen in round spermatids. No transcription of the Pgk-2 gene was detected in liver cells, but active transcription was detected in both pachytene spermatocytes and round spermatids.

Pgk-1 DH site 1 was clearly detected in premeiotic spermatogonia and was also evident in early primary spermatocytes, but was only very faintly detectable in pachytene spermatocytes (Fig. 4). This site was again clearly detectable in nuclei from round spermatids, but was absent in testicular spermatooza. To the extent that the presence of a DH site in the promoter region is indicative of ongoing transcription, these results are surprising in at least three respects. First, they indicate that maximum transcriptional repression of the Pgk-1 gene (and hence maximal XCI) occurs only in the pachytene stage of first meiotic prophase. Previous estimates (including our own) of the timing of XCI during spermatogenesis suggested this event occurs at the very beginning of meiosis (2, 3, 39, 40). While the inactivation process may indeed begin coincident with the initiation of meiosis, our results suggest that it does not reach a point of maximum transcriptional repression until the pachytene stage, which develops approximately 4 days after the initiation of meiotic prophase (41).

Second, our results indicate that transcriptional inactivation of the X chromosome during spermatogenesis, even at its maximum in pachytene spermatocytes, may never be as complete as that observed in somatic cells. The faint representation of Pgk-1 DH site 1 seen in DNA from pachytene spermatocytes could be due, in part, to the up to 10% contamination of this preparation with other cell types (earlier spermatocytes or later round spermatids) in which we have shown a clearly detectable site is present. However, we believe this result also indicates that while this site becomes maximally reduced at the pachytene stage, it never completely disappears. We (2) and others (10) previously showed that XCI in spermatogenic cells is not associated with stabilization of DNA methylation as it is in somatic cells. Now it appears that the complete disappearance of DH sites that is associated with genes on the inactivated X chromosome in somatic cells (7, 8, 16) may also fail to accrue during XCI in spermatogenic cells.

Finally, these results indicate that the inactivation mechanism becomes relaxed in postmeiotic round spermatids. This suggests that XCI during spermatogenesis is even more transient than previously thought. Recent results with the Ube-1X gene indicate that X-linked genes can indeed reactivate in round spermatids (42). To determine if the Pgk-1 gene undergoes a similar transcriptional reactivation, we used nuclear run-off assays to assay ongoing transcription in pachytene spermatocytes and round spermatids. Our results indicate that the relaxation of XCI in round spermatids may be associated with a slight increase of Pgk-1 transcription in these cells, but that this reactivation is very limited at most, and never returns to levels similar to those observed in somatic cells (Fig. 6).

A complete absence of DH sites was observed in both Pgk genes in spermatooza, which supports reports that all transcription is totally inactivated in male gametes (43), although some genes are known to retain an open chromatin domain in spermatooza (44).

2 P. Burgoyne, personal communication.
The apparent restriction of maximal transcriptional repression of the X-linked Pgk-1 gene to the pachytene stage may provide insight into the role of XCI in spermatogenic cells. Our results indicate XCI becomes maximal at the same stage during meiotic prophase when pairing and genetic exchange occur between transcriptionally active homologous autosomes. Unlike the autosomes, the X and Y sex chromosomes fail to pair or undergo genetic recombination outside the pseudoautosomal region during male meiosis. This mechanism apparently evolved to avoid disruption of the stable sex determination system in eutherian mammals which relies on the Y-specific Sry gene that maps outside the pseudoautosomal region (45, 46). Thus, repression of transcription from the X and Y chromosomes in spermatocytes could reflect the development of a chromatin state required to preclude pairing and recombination (39, 47). This could explain the very transient, and possibly incomplete, nature of XCI during spermatogenesis, since, unlike XCI in somatic cells, the function of this process is apparently not related to the regulation of gene expression per se.

Steady-state levels of many X-linked gene transcripts (e.g., Pgk-1, Pdha-1, Phka, Zfx, x) drop, while others (e.g., Hprt) remain fairly constant throughout spermatogenesis (3). Levels of mRNAs from other X-linked genes (e.g., Ube1X) are low in pachytene spermatocytes, but rise significantly again in round spermatids (42). This suggests that expression of most X-linked gene products is probably not deleterious to spermatogenesis as was previously suggested (48). Furthermore, the apparently active transcriptional state of the Pgk-1 gene in primary spermatocytes prior to the pachytene stage indicates the complete XCI is neither required for, nor associated with, the entry of spermatogenic cells into meiotic prophase.

These results correlate with previous results regarding expression of the Xist gene during spermatogenesis. The Xist gene, which is known to be a regulator of XCI in somatic cells (49), is also expressed prior to, and during, the period of XCI in spermatogenic cells (50–52). The apparent relaxation of XCI in round spermatids correlates with the previously observed decline in levels of Xist mRNA in these cells (50). Our nuclear run-off results suggest this relaxation does not result in a significant reduction of transcription of the Pgk-1 gene. This could be due to a lack of binding of one or more required transcription factors, and/or it may be related in part to changes in chromatin components that occur during spermiogenesis, as first transitional proteins and then protamines replace histones (53).

Three DH sites were detected in the Pgk-2 gene in expressing spermatocytes and spermatids. Faint representation of these three sites was also detected in a mixture of type A and type B spermatogonia, but not in a pure population of primitive type A spermatogonia. The occurrence of faint sites in the type A and type B spermatogonia is not likely due to contamination of this preparation with later Pgk-2-expressing spermatocytes, since spermatocytes have not begun to appear in the testes of mice at 8 days of age (18), and our previous attempts to detect Pgk-2 mRNA in these cells were negative, even when the reverse transcription-polymerase chain reaction was used (2, 3). The absence of Pgk-2 DH sites in primitive type A spermatogonia indicates these sites first appear in either type A or type B spermatogonia, up to 2 days prior to the initiation of Pgk-2 transcription in pachytene spermatocytes (41).

Our results for the Pgk-2 gene are in agreement with the general observation that DH sites typically appear prior to the actual initiation of transcription (54). The appearance of these sites also appears to occur just prior to, or at about the same time as, the initial appearance of binding activities of testis-specific nuclear proteins in the Pgk-2 enhancer region, to the extent the timing of these activities can be determined using gel shift experiments with developmentally staged testicular cells (15). Thus, we can now order putative regulatory parameters associated with the initiation of transcription of the Pgk-2 gene as follows: 1) demethylation of the 5′ half of the gene occurs in spermatagonia at 18.5–21.5 days postcoitum (55); 2) the appearance of DH sites in the Pgk-2 promoter/enhancer (this report) and 3) the appearance of nuclear proteins that bind to the Pgk-2 enhancer (15), both occur in either late spermatogonia or the earliest spermatocytes; and 4) the initiation of transcription occurs in primary spermatocytes at the preleptotene stage (2). Finally, the results from the nuclear run-off assays prove unequivocally that the Pgk-2 gene is actively transcribed at both meiotic and postmeiotic stages of spermatogenesis.

We conclude that the appearance and disappearance of DH sites in the 5′-flanking region of both Pgk genes correlates closely with their differential expression during spermatogenesis as examined by Northern blot, reverse transcription-polymerase chain reaction, and in situ hybridization analyses (2, 3, 56). The only exceptions to this strict correlation may include: 1) the reappearance of DH site 1 in the Pgk-1 gene in round spermatids in which transcription of the this gene was detectable at only minimal levels, and 2) the initial appearance of DH sites in the Pgk-2 gene in type A and/or type B spermatogonia, just prior to the actual activation of transcription of this gene in primary spermatocytes.

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