Distinct Promoters Determine Alternative Transcription of gpx-4 into Phospholipid-Hydroperoxide Glutathione Peroxidase Variants*

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A nuclear variant of phospholipid-hydroperoxide glutathione peroxidase (PHGPx, GPx-4) was considered to be derived from alternative pre-mRNA splicing in testis and to regulate sperm maturation. The genomic sequence of rat gpx-4 was established and investigated in respect to expression into the cytosolic, mitochondrial, and nuclear forms of PHGPx. In silico analysis suggested the presence of two distinct promoter regions, the upstream one leading to transcripts translating into cPHGPx or mPHGPx and the downstream one yielding nPHGPx. The promoter activity of both regions was verified by luciferase-based reporter constructs in A7r5 and H9c2 cells. The data reveal that the formation of nPHGPx is due to alternative transcription and not to alternative splicing. Transcripts encoding nPHGPx were most abundant in testis although not restricted to this organ. This observation points to a general role of the nuclear PHGPx variant in regulating cell division.

The family of glutathione peroxidases (GPxs)\(^1\) comprises different gene products with a conserved catalytic triad composed of (seleno)cysteine, tryptophan, and glutamine (1). They reduce hydroperoxides to corresponding alcohols at the expense of thiols, typically of glutathione (GSH). The rate constant for the reaction of hydroperoxides in the range of 10\(^5\) to 10\(^7\) M\(^{-1}\) s\(^{-1}\) is fast enough to cope with oxidant production in cells. Therefore, Se-GPx\(_s\) are commonly considered to play a key role in cellular antioxidant defense. The best studied members of the family are cellular glutathione peroxidase (cGPx\(_1\); EC 1.11.1.9), a tetrameric enzyme known since 1957 (2), and phospholipid-hydroperoxide glutathione peroxidase (PHGPx\(_s\); GPx\(_4\); EC 1.11.1.12), a monomeric variant discovered in 1982 (3). cGPx is a selenoprotein band of 34 kDa, detected in testis (23), has been identified as a PHGPx variant with an N-terminal extension that comprises a nuclear targeting signal (24). This sperm nuclei, PHGPx ("sn GPx" in Ref. 24), is proposed to be involved in chromatin condensation and thereby to determine the differentiation of spermatogenic cells into spermatozoa (24).

How the nuclear PHGPx (nPHGPx) is formed is still debated. The PHGPx gene is composed of eight exons that, depending on species, are spread over 3 to 4 kb of DNA. It is transcribed into mRNAs of different lengths (25–27), which lead to three different isoforms differing in their N-terminal extensions: a cytosolic one (cPHGPx), a mitochondrial one (mPHGPx), and nPHGPx. cPHGPx and mPHGPx are derived from the same exon (designated JA; see Fig. 1) by use of different translation stars, whereas the N-terminal extension of nPHGPx results from translation of an alternative exon (designated JB; see Fig. 1). This phenomenon was first explained as resulting from alternative pre-mRNA splicing by Pfeifer et al. (24). The concept of splicing was also followed through by others (28). In the meantime, Moreno et al. (26) provided strong evidence for the existence of a second promoter that is located in the first intron of (seleno)cysteine, tryptophan, and glutamine (1). They reduce hydroperoxides to corresponding alcohols at the expense of thiols, typically of glutathione (GSH). The rate constant for the reaction of hydroperoxides in the range of 10\(^5\) to 10\(^7\) M\(^{-1}\) s\(^{-1}\) is fast enough to cope with oxidant production in cells. Therefore, Se-GPx\(_s\) are commonly considered to play a key role in cellular antioxidant defense. The best studied members of the family are cellular glutathione peroxidase (cGPx\(_1\); EC 1.11.1.9), a tetrameric enzyme known since 1957 (2), and phospholipid-hydroperoxide glutathione peroxidase (PHGPx\(_s\); GPx\(_4\); EC 1.11.1.12), a monomeric variant discovered in 1982 (3). cGPx is a selenoprotein band of 34 kDa, detected in testis (23), has been identified as a PHGPx variant with an N-terminal extension that comprises a nuclear targeting signal (24). This sperm nuclei, PHGPx ("sn GPx" in Ref. 24), is proposed to be involved in chromatin condensation and thereby to determine the differentiation of spermatogenic cells into spermatozoa (24).

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and creates multiple transcription starts, including the alternative exon 1B, which is spliced to exons 2–7 and translated into nPHGPx. Attempts failed, however, to prove the activity of this alternative promoter region in reporter gene-transfected somatic cells (26, 27). This was interpreted by Borchart et al. (27) to support the original idea of alternative splicing of a transcript derived from the “joint basic promoter” upstream of exon 1A, which was Navone et al. (26), argue that the supposed alternative promoter is not recognized in somatic cells because it likely depends on transcription factors regulating nPHGPx expression specifically in germ cells.

We describe here reporter gene experiments revealing the activity of the homologous alternative promoter of rat gpx-4 even in somatic cell lines. We further detected nPHGPx-encoding transcripts not only in testis but also in somatic cells and tissues, which opens up the perspective of a potential role for nPHGPx in the regulation of cell division beyond germ line differentiation.

EXPERIMENTAL PROCEDURES

Cloning Rat gpx-4—Rat gpx-4 was isolated with rat Genome Walker Kits (Clontech, Palo Alto, CA) using the adaptor-ligated rat libraries prepared and processed according to the manufacturer’s instructions. The 5′-ends of the mRNA sequence from −1497 to +2891 was finally elucidated by PCR using pfu polymerase, genomic DNA of Wistar rats (TRIzol reagent, Invitrogen) and the Mini-kit (Qiagen, Hilden, Germany) and retrotranscribed into cDNA. PHGPx mRNAs were obtained by RACE cDNA amplification (SMART kit; forward, 5′-tgcacgaattctcagccca-3′). The RACE-ready cDNA samples of 1 μg each were purified (Plasmid Midi-kit, Qiagen) and quantified by measurements. Total RNA was again extracted using the RNeasy Mini-kit. Genomic contamination was ruled out as negative. The first 5′-RACE PCR were performed with reverse primers targeting exon 1A (5′-cagaggcaagccacagc-3′) or exon 1B (5′-cagagactgtgctgtctgctg-3′) to gain the cytosolic and mitochondrial or nuclear transcripts, respectively. For the second amplification, nested reverse primers for exon 1A or 1B (5′-tgctgctcaataagctggcagtacg-3′ or 5′-tgtaagaagctggaaagatg-3′) were used. The agarose gel-purified RACE products were TA-cloned and sequenced.

Amplification of Rat Testes PHGPx mRNAs—120 ng of retrotranscribed RNA was amplified (PCR master mix, Promega) from total RNA of liver, heart, testis, and H9c2 cells. RNA was prepared using the RNaseasy Mini-kit (Qiagen, Hilden, Germany) and retrotranscribed into 5′-RACE-ready cDNA samples of 1 μg each. The first 5′-RACE PCR were performed with reverse primers targeting exon 1A (5′-cagaggcaagccacagc-3′) or exon 1B (5′-cagagactgtgctgtctgctg-3′) to gain the cytosolic and mitochondrial or nuclear transcripts, respectively. The second amplification, nested reverse primers for exon 1A or 1B (5′-tgctgctcaataagctggcagtacg-3′ or 5′-tgtaagaagctggaaagatg-3′) were used. The agarose gel-purified RACE products were TA-cloned and sequenced.

Amplification of Rat Testis PHGPx mRNA—120 ng of retrotranscribed RNA was amplified (PCR master mix, Promega, Milano, Italy) in the presence of 8% dimethyl sulfoxide and 0.8 μM of the following primers for the mitochondrial transcript: reverse, 5′-gtgagaaagttgctgctg-3′, and forward, 5′-tccatgtgctgtctgctg-3′; or forward, 5′-aggggggggaagggcaagccacagc-3′, or forward, 5′-attaagctggctgtctgctg-3′; or forward, 5′-ccacagctgctgctgctg-3′; and for the nuclear transcript: reverse, 5′-atgacacgacacccgcagcagc-3′, or forward, 5′-ccacagctgctgctgctg-3′; or forward, 5′-ttgagaaagttgctgctg-3′; or reverse, 5′-ccctgctccatgtgc-3′. The annealing temperature was 59°C. Any genomic contamination of the retrotranscribed sample was ruled out by PCR with PCR-4-specific primers annealing to exons 1A and 4: forward, 5′-ctgacagctgtgctgtctgctg-3′; reverse, 5′-ctgacagctgtgctgtctgctg-3′. The real-time PCR measurements—Total RNA was again extracted using the RNAeasy Mini-kit. Genomic contamination was ruled out as described above. Only samples with an A260/280 nm ratio of 1.8 were accepted.

Standard plasmids were prepared by TA cloning of PCR products obtained from rat testis cDNA with the following primers: forward, 5′-ccctgctccatgtgc-3′, reverse, 5′-ccacagctgctgctgctg-3′ (for the cytosolic regulatory transcript); or forward, 5′-ctgagaaagttgctgctg-3′, reverse, 5′-ggtgagaaagttgctgctg-3′ (for the nuclear transcript). After propagation in Escherichia coli, plasmids containing the correct insert were purified (Plasmid Midi-kit, Qiagen) and quantified by A260 measurements.

**RESULTS**

Gene Organization—The overall organization of the rat PHGPx gene, gpx-4, which has been cloned here for the first time, appears to be essentially identical to that previously reported for mouse (31) and humans (32). It complies with the assumption that the coding region is constituted by 7 exons, the first one existing in two alternative forms that encode different transcript; the other positions shown were used for reporter gene constructs (see also Fig. 2). The red squares enclose the promoter areas as proposed by in situ analysis.

Six hundred ng of RNA was retrotranscribed using the Taqman reverse transcription reagents kit (Applied Biosystems, Branchburg, NJ). Amplification was performed on an ABI PRISM 7007 sequence detection system (Applied Biosystems, Foster City, CA) and analyzed by SYBR green PCR core reagents (PE Applied Biosystems, Warrington, UK). The amplification conditions for nuclear cDNA contained 300 ng M(r) 0.2 μm primers (forward, 5′-cctgctccatgtgc-3′; reverse, 5′-ggtgagaaagttgctgctg-3′) and serial dilutions of the retrotranscription mixture (1.5–15 ng of starting RNA). Standard reagents were adapted to a final volume of 25 μl. Amplification conditions were: 95°C, 10 min; 95°C, 15 s, and 63°C, 1 min for 40 cycles. The amplification conditions of the cytosolic/mitochondrial transcripts were identical except for the concentration of M(r) 0.2 μm, the primers (0.4 μm; forward, 5′-cttgagaaagttgctgctg-3′; reverse, 5′-ggtgagaaagttgctgctg-3′), and the annealing/extension temperature (65°C).

**Reporter Gene Experiments—**Fragments of gpx-4 were generated by PCR with genomic DNA as template and gpx-4-specific primers and cloned into the KpnI/BglII site of the promoterless pGL3-Basic luciferase reporter vector (Promega). The primers were designed to yield the fragments −547 to −30, −547 to +422, −313 to +422, −179 to +422, +39 to +422, +86 to +422, and +86 to +649. The accuracy of the constructs was verified by sequencing.

Cells were seeded onto 24-well plates, grown to 70% confluence, and transfected with 0.2 μg of the above constructs and appropriate control vectors (pGLBasic vector for negative control and pRL-TK vector expressing Renilla luciferase (Promega) for control of transfection efficiency) using ExGen 500 (MBI-Fermentas, St. Lech-Rot, Germany). Cells were transfected with 0.2 μg of one of the above experimental constructs or 0.2 μg of empty vector. After a 24-h expression period, cell lysates were assayed for luciferase activity by the Dual-luciferase Reporter Assay System (Promega) on a plate-reading luminometer (Fluoskan Ascent FL, Labsystem, Helsinki, Finland). Firefly luciferase activity of the experimental constructs was normalized by the Renilla luciferase activity obtained with the control vector pRL-TK and expressed as amplification factor in respect to the basic luminescence observed with pGLBasic/pRL-TK-transfected cells.

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A difference from the previously reported sequences of the N-terminal extension was observed. The sequence 12QCAGIRPYGP21 (24) is replaced here by 12CRQ14; this diversity was detected independently by others and thus is likely common. Otherwise, the total genomic sequence now established, −1497 to +2714 (not shown; GenBank accession no. AJ537598), is remarkably congruent with published cDNAs (25) and partial genomic sequences (24). Just the insertion of a gctccgg motif yielding the nuclear variant.

An in silico analysis of the entire rat gpx-4 sequence from −1497 to +2714 by the Genomatix PromoterInspector program (available at www.genomatix.de) suggested two distinct promoter areas (Fig. 1). The most upstream one corresponds in sequence and location to the promoter first identified by Huang et al. (35) in human GPX-4 and confirmed to be active by Maiorino et al. (22) and Borchert et al. (27). Being upstream of exon 1A, it may reasonably be presumed to regulate the products derived from this exon, i.e. cPHGPx and nPHGPx. The second promoter option is located in the first intron upstream of the alternative exon 1B. It is homologous to the mouse promoter postulated by Moreno et al. (26) to regulate the expression of nPHGPx, which depends on the transcription of exon 1B. This hypothetical promoter comprises 308 nucleotides that, according to MatInspector (36), contain 29 putative binding sites for transcription factors or regulatory proteins with a core similarity of 1 and an “optimized” matrix similarity. With identical criteria, a further 17 putative binding sites were identified in the region 340 nucleotides upstream of the putative promoter. In contrast to the corresponding region of the mouse gpx-4, no testis-specific regulatory elements were suggested in this area by the MatInspector program.

Typical splice sites that might yield an mRNA translating into nPHGPx, as suggested by Pfeifer et al. (24) and Borchert et al. (27), could not be detected irrespective of the assumed transcription start.

Proof of Second Promoter Activity—Left with no reasonable source to explain nPHGPx formation by alternative splicing and encouraged by the in silico prediction of the two promoters in gpx-4, we decided to reinvestigate the promoter activities with conventional luciferase-based reporter gene constructs and to test them in homologous, i.e. rat, mesenchymal cell lines.

Expectedly, the sequence −547 to −30 comprising the canonical upstream promoter only was active in both the embryonic rat heart myoblast line H9c2 and the smooth muscle cell line A7r5 (Fig. 2). If the whole stretch from −547 up to the translation start of exon 1B was lined to the reporter gene, activity declined to about 50% in both cell lines, suggesting that downstream elements bind inhibitory ligands. The removal of sequences upstream of the predicted promoter did not significantly affect the results. So far, the data is in line with the canonical promoter being localized in front of exon 1A. The constructs, starting at +39 and +86, however, which totally lack the canonical promoter, also showed significant activity in H9c2 cells, whereas in the A7r5 cells their activity was marginal. Furthermore, in both cell lines, increased activity was observed by including exon 1B almost completely into the construct lacking the canonical promoter (+86 to +649). These findings validate the prediction of the PromoterInspector program and, for the first time, unambiguously demonstrate that the hypothetical second promoter of gpx-4 is active in a cell type-dependent manner.

Detection of Exon 1B-derived Transcripts—As expected, the RACE technology applied to mRNA isolated from whole testis detected transcripts corresponding to both exons 1A and 1B (Table I). The 1B transcript extended 14 bases into the 5′-UTR, which agrees perfectly with the major transcription start region at −12 to −14 upstream of the AUG translation site of nPHGPx, worked out by Moreno et al. (26) for the mouse system. Surprisingly, however, an identical transcript could also be detected with mRNA isolated from heart and liver. This clearly indicates that the use of the alternate promoter is not restricted to germ line cells, and accordingly, nPHGPx is not a testis-specific protein.

In the H9c2 cells, which had been shown to use the alternate promoter efficiently, a similar and two additional RACE prod-

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\(^2\) R. Brigelius-Flohé, personal communication.
ucts were obtained, indicating additional transcription sites at −18, −73, and −108 relative to the nuclear translation start, which complies with the additional upstream transcription sites detected in the mouse system (26). Under our conditions, probing testis mRNA for the region upstream to exon 1A or 1B by reverse transcription PCR with suitable reverse primers and a series of forward primers nesting further 5′ to the uppermost transcription start sites identified for each exon product by RACE, a corresponding series of the correct-sized bands was observed (Fig. 3). This suggests that, in both cases, transcription may be initiated even further upstream than suggested by RACE experiments.

In agreement with previous observations (25, 33), some of the exon 1A-derived transcripts identified here (Table I) do not cover the translational start of the mitochondrial PHGPx. This observation leaves open the possibility that the expression of gpx-4 into mPHGPx or cPHGPx, respectively, is also determined by alternative transcription and not only by an alternative use of the translation start site.

Quantitative Transcript Analysis by Real-time PCR—Transcripts encoding the exon 1A-derived products cPHGPx and mPHGPx and the 1B-derived nPHGPx were detectable by real-time PCR in somatic cell lines and all tissues investigated, i.e. testis as well as brain, heart, liver, and kidney. In each case nPHGPx transcript levels were substantially lower than those encoding the cytosolic and mitochondrial forms (Table II). In terms of percentage of total PHGPx transcripts, the nPHGPx transcript varied between 17% in testis and 0.4% for the A7r5 cell line that had shown low activity of the PHGPx—suggesting the presence of alternate promoters. The data reveals that nPHGPx is most abundantly expressed in testis in terms of both, absolute and relative amounts, but transcription of gpx-4 into nPHGPx-encoding mRNA is not restricted to spermatogenic cells.

### DISCUSSION

The controversy about the mechanism of nPHGPx transcription can now be settled with a high degree of accuracy. The original proposal (24, 27, 28), which assumes nPHGPx to result from alternate splicing, conflicts with the 5′-UTR sequences of nPHGPx-encoding transcripts (Table I). None of them starts with a 5′-end corresponding to any known sequence upstream of exon 1A, and the genomic gpx-4 sequence upstream of the identified 5′-ends of the transcription starts do not contain any splice sites that could reasonably yield the sequences of the identified transcripts, as similarly argued by Moreno et al. (26) for the mouse system. Instead, multiple transcription start sites similar to those of the mouse system were identified here immediately and further upstream of the translation start site of exon 1B (Table I, Fig. 3), strongly supporting the concept that nPHGPx results from the use of an alternate promoter located in intron 1. The argument made in favor of the alternate splicing concept, that the intron region upstream of exon 1B does not display any promoter activity (27), is invalidated by the demonstration of such activity in H9c2 cells (Fig. 2). Furthermore, our data suggest the presence of downstream elements, increasing novel promoter activity, within exon 1B. The failure to detect the promoter activity of the intron region preceding exon 1B (26, 27) is likely explained by its relatively low and variable response in somatic cells, which is reflected in the relative and absolute low abundance of pertinent transcripts in tissues other than testis (Table II) and/or by the use of heterologous systems for reporter gene experiments in the previous work (26, 27). In short, therefore, translation of gpx-4 into nPHGPx instead of cPHGPx or mPHGPx is caused by the

### TABLE I

| Tissue/cells     | Uppermost 5′-end found |
|------------------|------------------------|
| Testis           | GGCTGCGTGAGAGGGAGACCCGCTGGGCTCCTGGGCGCCGATGAGCTGGGCGCTCTTCGCTTGTGCGCTTGGCTGGGTGCCACCATG |
| Heart            | GCCGAGATGCTCTGGGCGGCGTCTGAGCCGCTTTATATGAAAGACCACGACTCTGCTGGGCTCTGGCTGGCTGGCTGGGCTTGG |
| Heart, liver     | GGGGGCTGCTGAGGGGCTTATATTGAGAGGCCACGACTCTGCTGGGCTCTGGCTGGCTGGCTGGCTGGCTGGGCTTGG |
| H9c2             | GGGGGCGTGCTGACCCACCATG |
| H9c2             | GTGACCTGGGCTTATATTGAGAGGCCACGACTCTGCTGGGCTCTGGCTGGCTGGCTGGCTGGGCTTGG |
| H9c2, heart, liver | AGCAAGAGACACGGGCGAG |

### TABLE II

| Cell type/tissue | nPHGPx Ratio |
|------------------|--------------|
|                  | copying RNA | cPHGPx + mPHGPx | Ratio   |
| H9c2             | 132 ± 8     | 18,330 ± 2,356  | 0.007   |
| A7r5             | 84 ± 15     | 20,230 ± 1,888  | 0.004   |
| Brain            | 102 ± 1     | 7,515 ± 544    | 0.013   |
| Heart            | 16 ± 6      | 838 ± 176      | 0.019   |
| Liver            | 32 ± 15     | 3,625 ± 185    | 0.009   |
| Kidney           | 478 ± 16    | 17,418 ± 427   | 0.03    |
| Testis           | 18,524 ± 509 | 110,770 ± 6,116 | 0.17    |

**FIG. 3.** Reverse transcription PCR analysis of the PHGPx transcripts 5′-ends. The 5′-ends of the transcripts of exon 1A and 1B or mPHGPx and nPHGPx mRNA, respectively (lanes 1–4 and A–C), were probed with a series of forward primers annealing 5′ to the uppermost transcription start as identified by RACE technology (see below). As template retrotranscribed, total mRNA from testis was used. Reverse primers were within exon 2 for mPHGPx and at the boundary of exons 1B and 2 for nPHGPx, respectively. Lanes are as follows: 1, forward primer annealing to the gpx-4 position −50 to −33; 2, −81 to −64; 3, −166 to −149; 4, −313 to −290; A, +236 to +253; B, +174 to +191; C, +86 to +103. L marks the molecular weight ladder. For either transcript, no signal was detected using primers annealing further 5′ upstream than −313 to −290 and +86 to +103 (not shown). See “Experimental Procedures” for other details.
use of distinct transcriptional initiation sites under cell type-specific regulation by an alternate promoter and not by alternative splicing.

The abundance of gpX-4 transcripts in testes underscores the relevance of the PHGPx to spermatogenesis and spermiogenesis, and indeed all of the three different forms may be equally important. Morphological alterations of spermatozoa, as were reported for hemizygous mice (20) and clinical sperm samples of infertile patients (21, 37), are best explained by the role of PHGPx as a structural protein of the mitochondrial capsule in sperm. Although only cPHGPx were detected in the capsule material (16), both mPHGPx and cPHGPx may be considered to provide the raw material for the capsule formation, because these two forms can no longer be distinguished after processing. However the oligospermia consistently observed in transgenic animals (20), in severely selenium-deficient experimental animals (38), and in infertile males with low PHGPx content in sperm (21, 37) points to a distinct role for the active enzyme in earlier stages of spermatogenesis, the PHGPx form involved being unclear. The appearance of nPHGPx in late spermiogenesis (24, 26, 28) coincides with DNA condensation. The ability of PHGPx to act on -SH groups of protamine as alternate substrates (24, 39) prompted the idea that its nuclear variant is used for forming thiol groups as an alternate substrate of protamine thiol groups. This process is also considered to guarantee appropriate sperm function and arrest of proliferation, once the germ line cells have reached their final state of differentiation (24, 26).

Under consideration for this postulated role of nPHGPx in spermatogenesis, it ubiquitous expression documented here may offer a key to understanding the essential nature of PHGPx in embryonic development. Surprisingly, all efforts to obtain homozygous PHGPx (+/–) mice have failed thus far.3 This outcome of gene disruption experiments cannot be explained by any of the established roles of PHGPx. Knock-out of gpX-1 and gpX-2 yielded offspring with a mild phenotype. GPX-1 (+/–) mice developed normally and displayed no phenotype whatsoever as long as they were not oxidatively challenged. Appropriate controls demonstrated that GPX-1 is the GPx variant that dominates antioxidant defense (40), revealing that a disturbed hydroperoxide metabolism cannot account for prenatal lethality. Similarly, the gastrointestinal GPX-2 could be knocked out without impairing development. Even when crossed with GPX-1 (+/–) mice, the resulting double knock-out mice were vital and only later developed an intestinal inflammatory syndrome (41). It is thus extremely unlikely that PHGPx, which is less abundant than any of its congeners, is of vital importance because of its antioxidant potential. Modulation of cytokine responses, as reported for gpX-4 overexpression, cannot likely be reconciled with embryogenesis either, because it has been similarly reported for GPX-1 (42), and needless to say, the role of PHGPx as a structural protein of spermatozoa is irrelevant to embryonic development. Rather a unique role of PHGPx in cellular differentiation would be compatible with the outcome of the reverse genetics approach. The use of proteome -SH groups as an alternate substrate of PHGPx, as demonstrated for testis (39, 42), might be taken as a model for a more general principle of how the enzyme might regulate cell division, and the nuclear form would surely be predestined to fulfill such demands.

3 M. Brielmeyer, personal communication.