pound(s) bound tightly to the anion exchanger allowing the uncharged labeled glucose to pass through. The bound compounds that could be any of the phosphorylated compounds on the reaction sequence were sequentially eluted completely with 1 and 2 M HCl, which eluted 45% and 55% respectively. Use of stronger acid to completely elute the anion indicates that compounds of varying degrees of interaction were associated with the anion. This is expected as singly charged ions such as monophosphates are less tightly bound to the anion than the polyvalent ions such as pyrophosphates. Based on chromatographic analysis in which the R_f values of test samples were compared with those of authentic samples, it was concluded that the 1 M HCl effluents contained monophosphate compounds believed to be ribose phosphates while the 2 M HCl effluents contained PRPP. That the monophosphorylated compound was indeed a ribose phosphate was shown by comparing the R_f of the compound, which had been dephosphorylated, with that of glucose and ribose. The dephosphorylated compound cochromatographed with ribose. In this chromatographic system, ribulose and ribose had similar R_f values; therefore, it was not possible to state categorically that the compound was only ribose and not a mixture of ribose and ribulose. It was possible, however, to recycle the 1 M HCl effluents to PRPP to obtain additional ribose instead of attempting to separate ribose and ribulose. The 2 M HCl effluents on the other hand, contained a single compound, which was found to be PRPP. Dephosphorylation with alkaline phosphatase and inorganic pyrophosphatase produced a compound that cochromatographed with authentic ribose. It is worth noting here that the high salt concentration (approximately 2 M) in the final preparation may pose a problem in the enzyme assay. This can, however, be overcome either by starting synthesis with a radioisotope of high concentration or by concentrating the final product to achieve a higher concentration so as to use small volumes (microliter) for assays.

The labeled ribose also incorporated into the nucleic acid pool of the parasitic protozoan *T. brucei*, suggesting the presence of ribokinase that activated the free ribose by phosphorylation to ribose 5-phosphate. Previous study (4) that assayed for ribokinase activity in cell free extract had used [γ-^{32}P]ATP. This method cannot be used for *in vivo* tracing of the metabolic route as the label will be lost by dephosphorylation of the α-^{32}P-labeled R5P.

Further evidence that the 2 M HCl effluents were PRPP was obtained by converting the PRPP to GMP. The identities of the GMP and the dephosphorylated product, guanosine, were confirmed by thin-layer chromatography on cellulose developed in 1.25 M ammonium sulfate where the R_f of GMP and guanosine were 0.58 and 0.30 respectively. The labeled guanosine was specifically taken up by the bloodstream *T. brucei* and incorporated into the nucleotide pool.

The technique for the enzymatic synthesis of ribose labeled at the carbon atom is simple and easy to perform in a low budget laboratory. The advantage of synthesizing ^{14}C-labeled ribose from ^{14}C-labeled PRPP is that ribose of high purity is obtained with minimum manipulation. The problem though is low yield in that only 25% of the starting material was recovered as [5-^{14}C]ribose. However, even with the low yield, the synthesis is still justified as the commercial [5-^{14}C]ribose costs approximately 40× more than the laboratory synthesized labeled ribose.

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Quantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR): A Comparison of Primer-Dropping, Competitive, and Real-Time RT-PCRs

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Reverse transcription–polymerase chain reaction (RT-PCR)[2] is a sensitive technique for analysis of mRNA expression (1, 2). Though mainly used for qual-

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[2] Abbreviations used: RT-PCR, reverse transcription–polymerase chain reaction; MMP, matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol.

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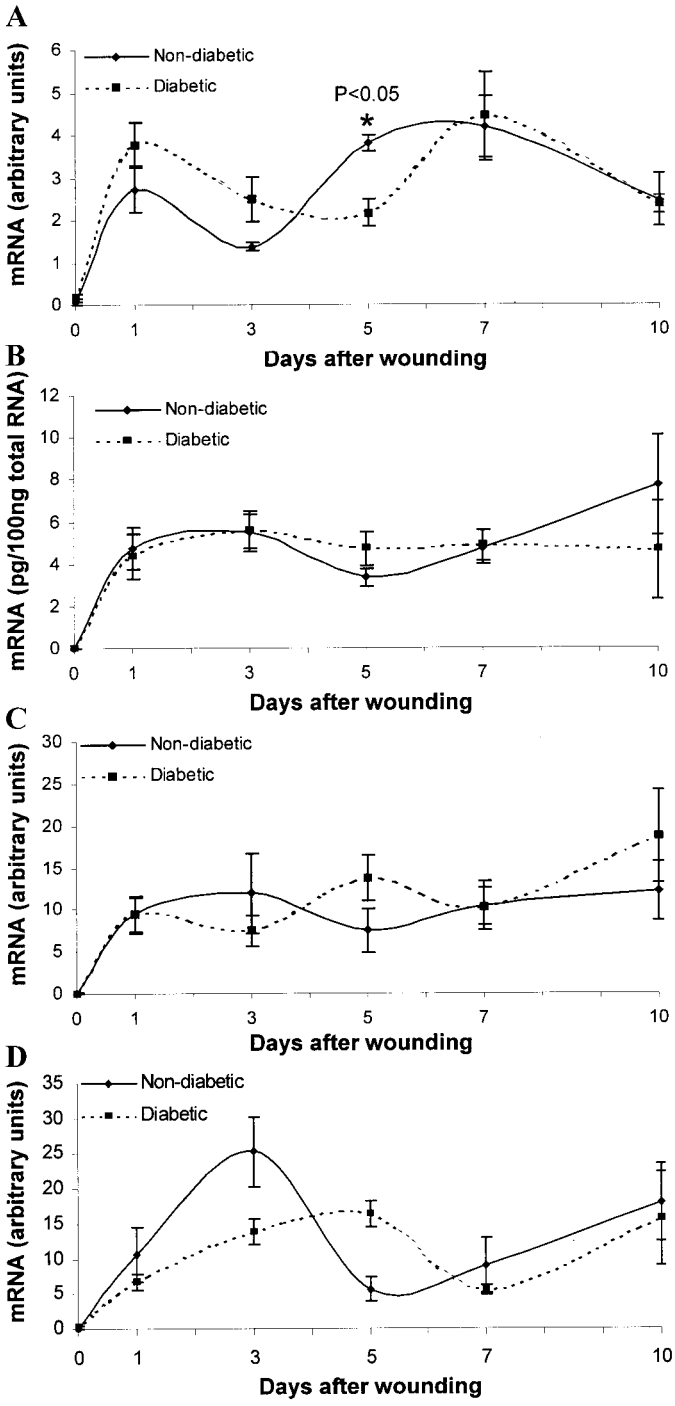

FIG. 1. MMP-13 mRNA expression time course during murine wound repair. (A) Primer-dropping RT-PCR was performed on all samples (100 ng total RNA/sample) using MMP-13 (32 cycles) and GAPDH (24 cycles) primers and the time course for MMP-13 generated after the samples had been normalized for GAPDH values. (B) Competitive RT-PCR was performed with MMP-13 primer on all samples (100 ng total RNA/sample) using a range of competitor concentrations (1, 5, 10, 20pg) and the time course for MMP-13 generated after the samples had been normalized for GAPDH values. (C) Real-time RT-PCR was performed for all samples (100 ng total RNA/sample) with primers for MMP-13 and GAPDH and the time course generated after the samples had been normalized for GAPDH values. (D) Real-time RT-PCR was performed for all samples (100 ng total RNA/sample) with primers for MMP-13 and 18S and the time course generated after the samples had been normalized for 18S values. Each point represents the mean value ± SE, where $n = 6$, with significant differences being highlighted (*Student's t test).

itative studies, several modifications of this method have been developed that allow quantitative analyses to be performed. Quantification by primer-dropping RT-PCR involves normalization to a housekeeping gene that is amplified within the same reaction as the target gene (3). Competitive RT-PCR, as the name implies, utilizes synthetic competitor RNA within the same reaction as the sample RNA (4). The newest form of quantitative RT-PCR is termed real-time RT-PCR, as measurements are taken throughout the reaction due to oligonucleotide cleavage by DNA polymerase that releases a fluorescent dye (5, 6).

During wound repair matrix metalloproteinases (MMPs) play a variety of crucial roles and it has been speculated that abnormal expression could contribute to impaired wound healing (7). This has been supported by studies showing elevated levels of several MMPs in chronic wounds compared to acute (8–10). The *diabetes* mouse (C57Bl/ksOlaHsd-db) shows impaired dermal wound healing compared to the nondiabetic littermates (11, 12) and so has been proposed as a model for impaired human wound healing. However, before this can be fully concluded comparisons to the human condition must be made between a number of wound healing factors, including the MMPs. Therefore, the aim of this study was to determine the expression profile of MMP-13 mRNA during dermal wound healing of *diabetes* and nondiabetic mice using all three quantitative RT-PCR methods. In addition to determining if any differences occur in MMP-13 expression between mouse types, and thus contributing to the impaired healing, this will enable direct comparisons to be made between the three methods. Additionally, a comparison was made using real-time RT-PCR between normalization for RNA loading with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA.

Materials and Methods

Mice. Under anesthesia 8- to 9-week-old C57Bl/ksOlaHsd-db mice (both sexes) were shaved and a single 6-mm full-thickness excisional wound made on each dorsolateral flank. Wounds were covered with BioClusive (Johnson & Johnson Medical Ltd.), which was attached with Vetbond tissue adhesive (3M), and the mice were housed individually. At defined points after wounding, the wound tissue and the surrounding

skin (1 mm from wound) were collected and the RNA was extracted.

Reverse transcription. One-hundred nanongrams of total RNA was used for each 100-μl RT [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ (Promega), 500 μM each dNTP (Bioline), 10 mM DTT (Promega), 50 units of RNase inhibitor (Promega), 2 μg N6-Random oligonucleotide (Amersham Pharmaceuticals), and 200 units of M-MLV-RT (Promega)]. The RT was incubated for 60 min at 37°C. Competitive RT-PCR had the additional step of adding competitor RNA to the RT mix (1, 5, 10, or 20 ng)—based on Wells *et al.* (4).

Primer-dropping PCR. A total of 2.5 μl of the RT reaction mix was used per 25-μl PCR reaction [20 mM Tris–HCl (pH 8.4), 50 mM KCl (Gibco BRL), 200 μM each dNTP, 1.5 mM $MgCl_2$ (Gibco BRL), 1.25 units of *Taq* polymerase (Gibco BRL), 500 pM 3′ primer, 500 pM 5′ primer (sequences upon request)]. PCR cycle conditions were 1 cycle at 94°C for 1 min; 32 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 2 min; and then 1 cycle at 72°C for 10 min. GAPDH primers added after 8 cycles—500 pM 5′ and 500 pM 3′ per reaction. The reaction was then allowed to go to completion. PCR products were analyzed by gel electrophoresis, captured onto computer by Grab-it' Version 2.55 software (Ultra Violet Products, Cambridge, UK), and analyzed using the 1D gel analysis program (Ultra Violet Products).

Competitive PCR. The methodology was essentially the same as that described for primer-dropping PCR; however, the addition of GAPDH primers at the end of cycle 8 was omitted and the cDNA used was that containing competitor as well as target RNA. Competitive PCR (10 ng only) was also performed with primers for GAPDH enabling GAPDH normalization and hence RNA loading normalization.

Real-time PCR. A total of 2.5 μl of the RT reaction mix was used per 25 μl PCR [1× TaqMan Mastermix (Applied Biosystems), 100 nM TaqMan probe, 200 nM of each primer (sequences upon request)]. TaqMan PCR conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed with the software provided with the TaqMan. TaqMan C_t values were converted to arbitrary units, followed by GAPDH normalization, by having a standard curve on each plate. Absolute quantification is possible if the standard curve utilizes a synthetic RNA corresponding to the target gene; however, here the standard was based on total RNA from mouse skin and so the analysis provided only relative quantification.

Statistical analysis. Data were considered to have a parametric distribution and so a Student's *t* test (two-tail) assuming unequal variance was performed for comparisons between data points. Pearson's correlation was performed to test for correlation between profiles. In all cases $P < 0.05$ was considered significant.

Results and Discussion

Repeated analysis of a single sample indicated that all three methods are highly reproducible, with real-time PCR showing the least variation with 1% (1.02 ± 0.014 arbitrary units), followed by primer dropping with 2% (4.69 ± 0.104 arbitrary units) and then competitive with 5% (7.78 ± 0.42 pg/100 ng total RNA).

All samples were analyzed by the three PCR methods and the resultant values were used to generate a time course of MMP-13 for each method [Figs. 1A (primer dropping), 1B (competitive), and 1C (real-time)]. All methods gave similar profiles, with no expression being detected in unwounded skin; however, following wounding MMP-13 mRNA expression became detectable from day 1 onward, with relatively small fluctuations in levels. Comparisons between methods indicated only minor variation in mRNA profile trends for real-time and competitive PCR methods (showing significant correlation, $P = 0.003$, $R^2 = 0.5909$; Pearson's), while primer dropping did not show significant correlation to either of the other methods. A number of reasons could be contributing to the greater variation seen for the primer-dropping method. Primer-dropping is the only technique where it is crucial that measurements are taken within the exponential phase of the PCR (2), and so is most prone to errors due to readings outside the range. Additionally, opening the reaction tube midcycle and the interaction of two primer sets could also affect the PCR reaction.

Although real-time and competitive RT-PCR methods generated the same mRNA profiles, real-time is preferable to competitive RT-PCR for a number of reasons. In addition to showing a higher degree of reproducibility the methodology is simpler and faster, without the need for post PCR processing and image analysis which may introduce systemic errors. It also possesses a greater detection range for target cDNA (5) and a higher degree of specificity, utilizing three sequence specific oligonucleotides (primers and probe) compared to the two (primers only) of competitive PCR. One disadvantage of the real-time method used here is that the quantification was relative, compared to the absolute values obtained with the competitive method. However, real-time can perform absolute quantification if a synthetic target RNA is available for calibration (13). Additionally, real-time PCR requires the use of a specialized real-time PCR machine that may not be accessible to all researchers.

The use of a housekeeping gene to normalize for RNA loading during RT-PCR is a well-established technique. However, several groups have reported that

the expression of these genes are variable between cell lines, tissues, and stages of the cell cycle (14, 15), thus questioning the validity of using such genes for RNA normalization. To test this, in addition to normalizing for GAPDH (Fig. 1C), real-time samples were also normalized for 18S rRNA (Fig. 1D). Comparison of the two MMP-13 profiles showed a similar trend and significant correlation ($P = 0.006$, $R^2 = 0.5437$; Pearson's). As normalization to two separate genes gave significantly similar profiles it can be concluded that measured differences reflect true changes in expression rather than RNA loading. Additionally, for dermal wound healing at least, normalization for RNA loading can be via GAPDH or 18S rRNA levels.

The observed expression profile is that which would be expected from a collagenase performing a role similar to that of MMP-1 in humans. Additionally, *in situ* hybridization has shown MMP-13 to be localized directly underneath migrating keratinocytes, colocalized with MMP-10, and occasionally in fibroblast-like cells (16). This again supports the idea that it is acting in the mouse in the same fashion as MMP-1 in human skin wounds. Within human wound healing MMP-1 is essential for keratinocyte migration and hence reepithelialization; therefore, MMP-13 may be playing a similar role in the mouse. MMP-13 had been considered to be the only collagenase in the mouse, though the recent discoveries of two mouse MMP-1-related genes (17) and MMP-8 (18, 19) have revealed the greater complexity of the mouse MMP family and highlighted the need to investigate these other collagenases during murine wound healing. As no difference in expression between *diabetes* and nondiabetic mice was detected for MMP-13 it is unlikely to be a contributing factor to the impaired healing.

Conclusion

Although all three types of quantitative RT-PCR produce a similar mRNA expression profile, the real-time and competitive RT-PCR methods are the most reliable as they produce significantly similar results. Of these techniques real-time RT-PCR is most favorable mainly due to the easier methodology. Additionally, it has been shown that although MMP-13 expression is upregulated following wounding, and so is likely involved during the healing process, no differences occur in expression between the healing impaired *diabetes* mouse and its nondiabetic littermate and so is unlikely to contribute to the impaired healing.

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Utility of Single-Strand Conformation Polymorphism Analysis Was Improved by Low-Cross-Linking Polyacrylamide Gel and Quick Low-Background Silver Staining

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Analysis of single nucleotide polymorphisms (SNPs)[2] of known genes has been focused on the molecular genetics to understand each genetic variation and mutation. One of the methods for screening SNPs, single-strand conformation polymorphism (SSCP), needs only electrophoresis apparatus (1), usually radioisotope labeling and a relatively large gel. However, careful procedures and special facilities are needed to handle them for experiments. Though silver staining had been used for SSCP analysis, it was not possible to preserve the gels for the comparison of SSCP patterns because of the cracking of gels during drying. In this paper, we presented a simple and quick method for SSCP analysis by silver staining using low-cross-linking polyacrylamide gel under low background without cracking during drying. Additionally, it is possible to elute the ssDNA from gel for further analysis.

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[2] Abbreviations used: SNP, single nucleotide polymorphisms; SSCP, single-strand conformation polymorphism; PDA, piperazine diacrylamide.

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The composition of SSCP gel (14 cm × 14 cm × 1 mm) was 16.8 ml of 30% acrylamide solution (acrylamide:piperazine diacrylamide (PDA) (Bio-Rad) 30:0.135), 2.4 ml of a 10× SSCP electrophoresis buffer (250 mM Tris, 1920 mM glycine), 4.8 ml of 50% glycerol, 162 μl of 10% ammonium persulfate, and 27 μl of TEMED. The electrophoresis buffer was a 1× SSCP electrophoresis buffer (1).

The DNA (10 ng) was mixed with 5 vol of 90% deionized formamide containing 20 mM EDTA and 0.1% bromphenol blue. The mixture was heated at 80°C for 5 min and cooled on ice.

Polymerized acrylamide gel were electrophoresed at a constant 450 V constant between 4 and 30°C after prerunning at 50 V for 10 min. The gel was removed from glass plates and fixed in 10% trichloroacetic acid for 10 min. The gel was twice washed in water for 5 min for after refixing in 50% methanol. The gel was kept at 4°C in 0.1% silver nitrate for 20 min and washed twice with water for 1 min. The bands were made visible in developing solution (2% sodium carbonate and 0.04% formaldehyde). In this process, if the color of the solution turned yellow, it had be changed. Color developing was stopped by a stopping solution (25% methanol, 7.5% acetic acid). The gel could be reserved in cellophane membrane for drying after placing it in 10% glycerol for 5 min.

Figure 1 shows the cracked gel during drying by gel dryer (Model 543, Bio-Rad). High-cross-linking gel (Fig. 1A) was cracked during drying, but no cracking in the low-cross-linking gel (Fig. 1B). Figure 2 shows the silver-stained SSCP low-cross-linking gel. The bands were clearly separated and there was no cracking after drying. Figure 2A is the silver-stained gel containing low-bispolyacrylamide gel. Figure 2B is the low-PDA polyacrylamide gel. The bands were separated fairly well on a low-PDA polyacrylamide gel with a significantly lower background. The reasons for lower background and better separation were that PDA did not contain amide which caused the background on silver staining, and the gel containing PDA was stronger than the gel containing bisacrylamide (2). Figure 2C is the example of SSCP analysis of ribulose-1,5-bisphosphate carboxylase oxygenase activase from rice. The PCR products were analyzed after the digestion by restriction enzymes (*Acc*II, *Alu*I, *Hha*I, and *Hae*III). We identified polymorphism in the gene of ribulose-1,5-bisphosphate carboxylase oxygenase large subunit in rice mutant by this method (data not shown).

There are many advantages of this system. First, it allows direct comparison of the SSCP patterns. X-ray film must be used for radioisotopes or fluorescent dye labeled for SSCP analysis. Previously, silver-staining SSCP gel could not be compared directly because of cracking during drying. Next, glutaraldehyde is not used in this silver staining and it was possible to elute