Quantitative Analysis of Protein-DNA Associations in Vivo Using Real-Time PCR

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Introduction

The chromatin immunoprecipitation (ChIP) assay has been used in recent years to identify the association of histone complexes and transcription factors with specific genomic DNA targets in cells of *Drosophila* or yeast. As shown in Figure 1, the technique first involves the treatment of cells with formaldehyde or ultraviolet light to covalently crosslink proteins to DNA. The chromatin from the cells is then isolated and fragmented (by restriction digestion or sonication), and the protein of interest is immunoprecipitated using a specific antibody. The coimmunoprecipitated DNA fragments are then liberated from the protein by treatment with proteinase K, and the DNA fragments are identified by Southern blotting or PCR (Kuo and Allis 1999). More recently, studies have established the utility of the ChIP assay in studying histone complex associations and transcription factor binding in mammalian cells as well (Boyd and Farnham 1999). However, a potential barrier to the quantitative study of some mammalian transcription factors by the ChIP assay is their relatively low level of expression in cells, coupled with the rather large size of the mammalian genome compared to yeast or *Drosophila*. Southern blot detection of DNA does not offer the necessary sensitivity for such studies, nor is traditional PCR easily amenable to quantitation. However, we demonstrate here that real-time PCR with continuous SYBR Green I fluorescence monitoring (Morrison et al. 1998) offers the level of sensitivity and quantitation necessary to study transcription factor-DNA interactions in the context of the mammalian genome.

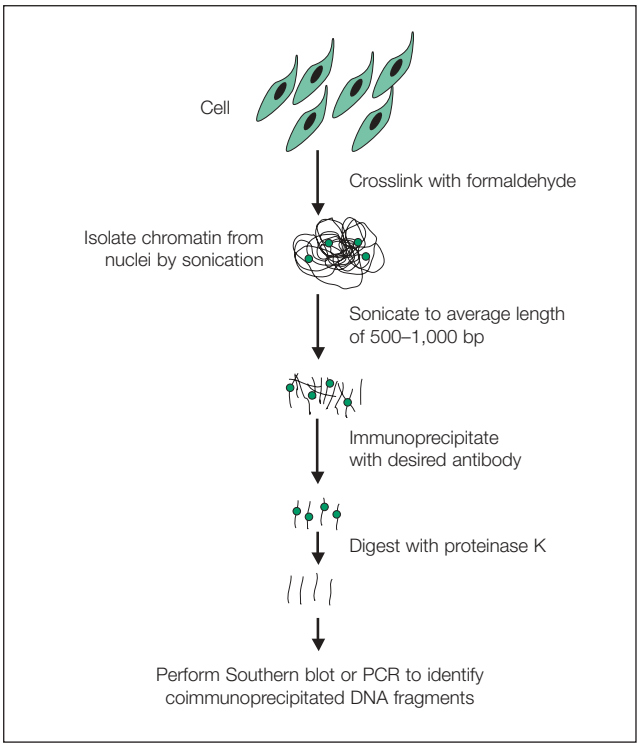

Fig. 1. Chromatin immunoprecipitation (ChIP) assay. The general scheme of the chromatin immunoprecipitation assay is shown.

The mammalian transcription factor Pdx1 (also known as IPF1) is expressed almost exclusively in β-cells in the mature mouse pancreas. β-cells express a unique set of genes, including insulin, glucose transporter type 2 (Glut2), islet amyloid polypeptide (IAPP), and glucokinase (GK). Several functional studies have implicated a direct role for Pdx1 in the specific activation of these genes (Ahlgren et al. 1998). However, none of these functional studies have established

whether Pdx1 is involved in a transcriptional complex that directly controls the expression of these genes. Since chromatin structure within a nuclear environment can influence both the binding of transcription factors to specific DNA targets and the resultant transcription rate, we cannot know from just functional and in vitro binding studies which genes Pdx1 natively regulates. To determine which promoters Pdx1 might directly regulate in vivo, we performed ChIP assays using Pdx1 antiserum and chromatin from Pdx1-transfected NIH 3T3 cells (NIH 3T3/Pdx1) and from empty vector-transfected NIH 3T3 cells (NIH 3T3/EV). Immunoprecipitated DNA fragments were detected by quantitative real-time PCR using SYBR Green I and the Bio-Rad iCycler iQ™ system.

Method

Chromatin immunoprecipitation assays using NIH 3T3/Pdx1 and NIH 3T3/EV cells were performed as previously described (Kuo and Allis 1999). Primers for the PCR reactions were designed using the MacVector software package (Accelrys), and are presented in the table. To demonstrate that promoter occupancy was independent of the specific region of the promoter amplified, we examined two different regions of the glucagon promoter, designated as Gluc(1) and Gluc(2). These two regions are located approximately 300 bp apart in the proximal glucagon promoter. For real-time PCR reactions, 3 µl of each immunoprecipitated sample was used as template in 25 µl reactions containing 3 mM $MgCl_2$, 0.25 mM dNTPs, 10 pmol primers, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen), and a 1:75,000 dilution of SYBR Green I dye (Molecular Probes). Thermal cycling was performed for 40 cycles using an iCycler iQ system, and continuous SYBR Green I fluorescence monitoring was performed according to Bio-Rad protocols. Cycling parameters for all amplifications were 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C, for 30 cycles. Amplified promoter fragments were subcloned into the T/A cloning vector pCR2.1 (Invitrogen) according to the manufacturer's protocol, and 3–4 clones from each PCR reaction were sequenced to confirm the identity of the amplified fragment.

The relative proportions of coimmunoprecipitated promoter fragments were determined based on the threshold cycle (C_T) value for each PCR reaction. The C_T value is defined as the cycle at which fluorescence rises to 10 times above the mean standard deviation of the background levels in all reaction wells. For every promoter studied, a ΔC_T value was calculated for each sample by subtracting the C_T value for the input (sample prior to immunoprecipitation) from the C_T value obtained for the immunoprecipitated sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value for the sample immunoprecipitated with Pdx1 antiserum from the ΔC_T value for the corresponding control sample immunoprecipitated with normal rabbit serum. Fold differences (Pdx1 ChIP relative to control ChIP) were then determined by raising 2 to the $\Delta\Delta C_T$ power. The equation used in these calculations is:

Fold-difference
(Pdx1 ChIP relative to control ChIP) = $2^{[\Delta C_T(\text{control}) - \Delta C_T(\text{Pdx1})]}$

where $\Delta C_T = C_T(\text{immunoprecipitated sample}) - C_T(\text{input})$

For every promoter fragment analyzed, each sample was quantitated in duplicate on at least two separate occasions, and from at least two independent immunoprecipitations, to give a minimum of four independent quantitations of each sample for each promoter. Quantitations were corrected to account for 10% inputs. Mean ± SD values were determined for each fold difference, and these values were subsequently used in two-tailed paired Student's t tests to determine statistical significance. A melt-curve analysis was performed for each sample after PCR amplification, to ensure that a single product of expected melt-curve characteristics was obtained.

Table. Primer sequences used for PCR amplification of promoter fragments.

Promoter	Primer	Sequences
Albumin	Forward	5'-TGGGAAAACTGGGAAAACCATC-3'
	Reverse	5'-CACTCTCACACATACACTCCTGCTG-3'
Mouse I insulin	Forward	5'-TCAGCCAAAGATGAAGAAGGTCTC-3'
	Reverse	5'-TCCAAACACTTGCCTGGTGC-3'
Glut2	Forward	5'-ATCTGGCTCCGCACTCTCATCTTG-3'
	Reverse	5'-CCCTGTGACTTTTCTGTGTCTTAGG-3'
IAPP	Forward	5'-TCACCCACACAAAGGCACTCAG-3'
	Reverse	5'-GGTTTCATTGGCAGATGGAGC-3'
Gluc(1)	Forward	5'-TCCAAACTGCCCTTTCCATTC-3'
	Reverse	5'-ATGATTTCACTCGCCCACTCAC-3'
Gluc(2)	Forward	5'-CGTAAAAAGCAGATGAGCAAAGTG-3'
	Reverse	5'-GAACAGGTGTAGACAGAGGGAGTCC-3'
Pax4	Forward	5'-CCAACGATCCAGGCTCTACATC-3'
	Reverse	5'-CGGGTTTGGGGCTAATTGTCC-3'
Pdx1	Forward	5'-TGGCTCGGGAAGGCTCTTG-3'
	Reverse	5'-CCATCAGGTGGCTAAATCCATTATG-3'
GK	Forward	5'-TGATAGGCACCAAGGCACTGAC-3'
	Reverse	5'-GCAGAAAACTGGGACTGATTGC-3'

Results and Discussion

Previous studies have documented that Pdx1 binds in vitro specifically to promoter elements containing the TAAT motif (Watada et al. 1996, Ohneda et al. 2000). However, none of these studies have examined directly the specificity of promoter binding in vivo, where the complexity of chromatin structure might physically hinder accessibility to some promoter elements but not others. We used the ChIP assay (Figure 1) to directly assess the binding of Pdx1 to promoter elements in vivo in Pdx1-transfected NIH 3T3 cells (NIH 3T3/Pdx1). As a negative control for the immuno-precipitations, we performed identical experiments in NIH 3T3 cells transfected with an empty vector (NIH 3T3/EV). By coupling the ChIP assay to real-time PCR using SYBR Green I and the iCycler iQ detection system, we were able to reproducibly quantitate relative occupancy by Pdx1 to several β-cell specific and nonspecific promoters.

Immunoprecipitated samples were subject to quantitative real-time PCR using primers to amplify the β-cell-specific mouse I insulin, Glut2, IAPP, glucokinase, Pdx1, and Pax4 gene promoters, and the non-β-cell-specific glucagon and albumin gene promoters. Primer pairs (see table) were designed to amplify specific promoter regions that are believed either to be bound and regulated by Pdx1 (promoters for mouse I insulin, Glut2, IAPP, Pdx1, Pax4, and glucokinase genes) or to contain important regulatory regions with potential Pdx1 binding sites (promoter for glucagon gene). As a negative control for binding, the albumin promoter fragment was chosen as a random genomic element containing Pdx1 binding sites, but not believed to be regulated by Pdx1. The promoter fragments were amplified as homogeneous products of expected sizes, as shown in Figure 2.

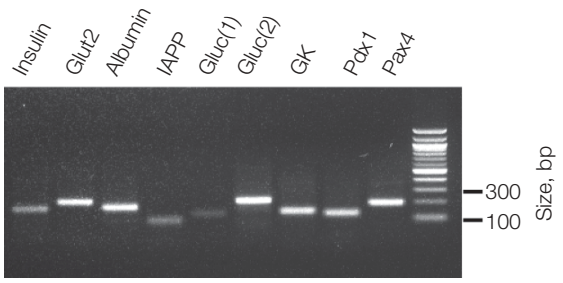

Fig. 2. PCR amplification of promoter fragments. Agarose gel (2%) stained with ethidium bromide showing products of amplification using primers shown in Table 1. Template was sonicated NIH 3T3 genomic DNA.

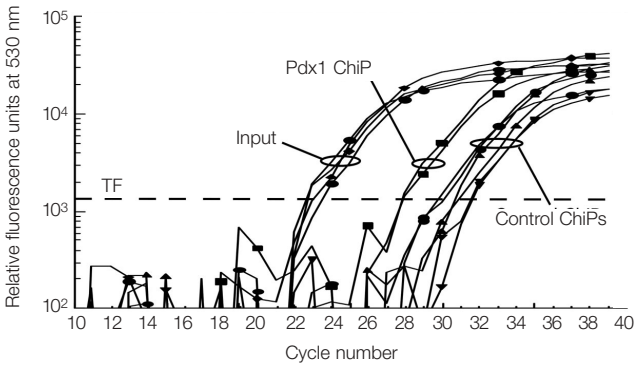

Fig. 3. Quantitative real-time PCR. Real-time PCR profiles for the amplification of the mouse I insulin promoter are shown for a representative ChIP assay in which chromatin from NIH 3T3/Pdx1 and NIH 3T3/EV cells was immunoprecipitated using either Pdx1 antiserum or normal rabbit serum. The data (shown in duplicate determinations) demonstrate the early exponential increase in fluorescence at 530 nm as a result of SYBR Green I binding to the amplifying insulin promoter fragment. "Pdx1 ChIP" represents duplicate curves from chromatin from NIH 3T3/Pdx1 cells that has been immuno-precipitated with Pdx1 antiserum; "Control ChIPs" represents duplicate curves from immunoprecipitations from NIH 3T3/Pdx1 chromatin using normal rabbit serum and from NIH 3T3/EV chromatin using Pdx1 antiserum or normal rabbit serum. "Input" represents curves from total genomic DNA from NIH 3T3/Pdx1 and NIH 3T3/EV cells prior to immunoprecipitation. C_T, the cycle at which the amplification curve reaches threshold fluorescence (TF), is used to determine the relative amount of promoter in each sample as detailed in Method.

As an example, Figure 3 shows the results (in duplicate determinations) of the amplification of the mouse I insulin promoter from NIH 3T3/Pdx1 and NIH 3T3/EV cells following ChIP. The data demonstrate that the NIH 3T3/Pdx1 chromatin that has been immunoprecipitated using Pdx1 antiserum reaches the threshold approximately three cycles before control immunoprecipitations (which included NIH 3T3/Pdx1 chromatin immunoprecipitated with normal rabbit serum and NIH 3T3/EV chromatin immunoprecipitated with Pdx1 antiserum or normal rabbit serum). This finding ($\Delta\Delta C_T$ = 3) implies an approximately 8-fold (i.e., 2^3) enrichment of insulin promoter in the NIH 3T3/Pdx1 immunoprecipitation compared to NIH 3T3/EV immunoprecipitations. Each value was normalized to the total input DNA (i.e., total genomic DNA), to correct for variations in input quantity of DNA prior to immunoprecipitation (as detailed in Method). The results shown in Figure 4 summarize the real-time PCR results for each of the promoters considered in this study.

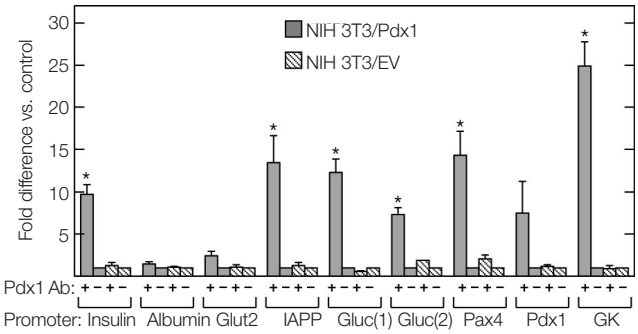

Fig. 4. Relative promoter-Pdx1 associations in vivo in NIH 3T3 cells. The data shown are derived from quantitative real-time PCR analysis of each of the promoters shown at the bottom, as detailed in Methods. Chromatin from NIH 3T3/Pdx1 and NIH 3T3/EV cells was immunoprecipitated with either Pdx1 antiserum (Pdx1 Ab^+) or with normal rabbit serum (Pdx1 Ab^-). The fold difference value compares the [Pdx1 Ab^+] sample to the corresponding [Pdx1 Ab^-] control sample (defined as "1"). In all cases, data represent the mean of duplicate determinations from each of three independent immunoprecipitations (a total of six determinations). An asterisk indicates that the value is significantly different ($p < 0.05$) from the control value.

Figure 4 demonstrates that in NIH 3T3/Pdx1 cells, Pdx1 shows 8–25-fold greater association with the insulin, IAPP, glucokinase, glucagon, and Pax4 promoters than in controls. While an approximately 7-fold greater association of Pdx1 with the Pdx1 promoter was observed, this was not statistically significant (P = 0.07). Pdx1 displays no statistically significant association with the Glut2 and albumin promoters, notwithstanding that these promoters contain potential high-affinity binding sites for Pdx1 based on in vitro binding assays. Moreover, the Glut2 promoter is believed to be a direct binding target for Pdx1 based on functional assays in cell lines (Waeber et al. 1996). Thus, our studies suggest that the DNA binding properties of Pdx1 in vitro do not necessarily predict its binding properties on chromatin templates in vivo. As expected in these studies, NIH 3T3/EV cells show no significant coimmunoprecipitation of any of the promoters studied.

In conclusion, our studies introduce a powerful technique (ChIP assay followed by real-time PCR) for quantitative examination of the association of proteins with DNA in vivo. Our results demonstrate that protein-DNA associations in vivo are governed not only by the intrinsic affinity of specific proteins for DNA, but also by the structure of chromatin, which may physically restrict accessibility to some DNA sequences.

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