Published online July 13, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 12 ***e104***
doi:10.1093/nar/gnh097

ChIP Display: novel method for identification of genomic targets of transcription factors

Artem Barski[1] and Baruch Frenkel[1,2,*]

[1]Department of Biochemistry and Molecular Biology and [2]Department of Orthopaedic Surgery, Institute for Genetic Medicine, Keck School of Medicine at the University of Southern California, 2250 Alcazar Street, Los Angeles, CA 90033, USA

Received May 25, 2004; Revised and Accepted June 18, 2004

ABSTRACT

Novel protein–DNA interactions in mammalian cells are traditionally discovered in the course of promoter studies. The genomic era presents opportunities for the reverse; namely, the discovery of novel target genes for transcription factors of interest. Chromatin immunoprecipitation (ChIP) is typically used to test whether a protein binds to a candidate promoter in living cells. We developed a new method, ChIP Display (CD), which allows genome-wide unbiased identification of target genes occupied by transcription factors of interest. Initial CD experiments pursuing target genes for RUNX2, an osteoblast master transcription factor, have already resulted in the identification of four genes that had never been reported as targets of RUNX2. One of them, *Osbpl8*, was subjected to mRNA and promoter–reporter analyses, which provided functional proof for its regulation by RUNX2. CD will help to assemble the puzzle of interactions between transcription factors and the genome.

INTRODUCTION

One of the most important aspects of gene regulation is the interaction of transcription factors with genomic *cis*-acting elements. Until recently, new binding sites for transcription factors have been discovered primarily during promoter studies focusing on known genes. Such studies typically include sequence analysis for consensus binding sites, electromobility shift assays, promoter–reporter analyses and chromatin immunoprecipitaion (ChIP) experiments. These approaches are skewed towards the studied genes, and specifically towards binding sites located in their proximal 5′ flanking sequences. A less biased approach for the discovery of transcription targets is manipulating the activity of transcription factors followed by gene expression analyses, such as RNA differential display (1) or expression microarrays. However, the interpretation of such experiments can be problematic. First, it is generally difficult to tell whether a responsive gene is a direct or an indirect target of the transcription factor of interest. Second, in experiments involving overexpression of transcription factors, the response of some genes may be forced by exaggerated concentrations of the transcription factor, resulting in physiologically insignificant results. Third, such studies do not provide information on the location of the *cis*-acting regulatory elements. Last, expression studies are unable to disclose genes, which bind the transcription factor of interest without influencing the respective mRNA levels under the experimental conditions used, due to, e.g. compensatory mechanisms or absence of co-activators that may be present under different conditions.

The approach described in this paper utilizes ChIP, i.e. immunoprecipitation of DNA fragments bound by a transcription factor in normally functioning living cells. The question typically addressed in ChIP experiments is whether a candidate genomic site is occupied by a given transcription factor. Occupancy is assessed by subjecting the immunoprecipitated material to PCR analysis and comparing the signal obtained for the candidate target versus that obtained for a random locus. It is tempting to assume that novel transcription factor binding sites can be simply cloned from the immunoprecipitated material. However, such 'ChIP cloning' is difficult due to the overwhelming excess of non-specifically precipitated DNA fragments. Still, several investigators have developed ChIP-based approaches to identify novel targets for transcription factors either by enhancing enrichment for targets via a second round of ChIP, screening the library of imunoprecipitated DNA for ability to bind the transcription factor *in vitro*, or hybridization of immunoprecipitated DNA to microarrays (2–4). The pros and cons of these approaches, as well as *ChIP Display*, introduced in the present paper, are addressed in the Discussion.

RUNX2 (a.k.a. CBFA1, AML3 and PEBP2αA) belongs to the *runt* DNA-binding domain family of transcription factors. It is preferentially expressed in osteoblasts (5) and partially mediates the osteogenic action of bone morphogenetic proteins (6,7). RUNX2 transcriptionally regulates several osteoblast marker genes including osteocalcin, collagen type I and osteopontin (6,8,9). In doing so, it interacts with other proteins, including CBFβ (10), p300 (11), HDAC6 (12), RB (13), AP1 (14), STAT1 (15) and LEF1 (16). Mice heterozygous for *Runx2* display a phenotype resembling cleidocranial dysplasia, a human autosomal dominant skeletal disorder characterized by failed closure of cranial sutures and lack of clavicles (17,18). Complete ablation of *Runx2*

*To whom correspondence should be addressed. Tel: +1 323 442 1322; Fax: +1 323 442 2764; Email: frenkel@usc.edu

results in absence of bone formation, attributable to maturational arrest of osteoblast differentiation (5). However, it is difficult to explain the osteogenic property of RUNX2 and the bone phenotype of the *Runx2* knockout mice based on the known RUNX2 targets alone. Thus, like many other transcription factors, RUNX2 is involved in an important physiological process, but the target genes it regulates are largely unknown.

Here we report the development of ChIP Display (CD), a novel method that allows the discovery of novel transcription factor binding sites. The usefulness of the method is demonstrated by our initial CD experiments, which have already led to the identification of four novel RUNX2 target genes.

MATERIALS AND METHODS

Cell culture

A subclone derived from the MC3T3-E1 osteoblastic cell line (19) was used for ChIP assay and EMSA. Cells were maintained in α–MEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Starting at confluency (day 3), 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid (Sigma) were added to support differentiation. ST2 cells (Ricken) were maintained in RPMI1640 supplemented with 10% FBS. For transfection, the medium was changed to D-MEM and then to differentiation medium as above. For RNA collection, the RPMI1640 was changed to differentiation medium as above starting at confluency (day 3).

ChIP Display

ChIP was performed essentially as previously described (20), except salmon sperm DNA was substituted by bacterial tRNA (Roche) for preadsorbtion of the A/G PLUS-agarose beads (Santa Cruz). One MC3T3-E1 plate (100 mm, day 8) was used for each immunoprecipitaton. Typical enrichment was 10 to 20-fold, estimated as the ratio of signals from *collagen* versus *insulin* (Figure 4 and supplementary Figure 2). Approximately one-fifth of the immunoprecipitated DNA was subjected to CD. The DNA was treated with SAP (Promega) in 1× React1 buffer (Invitrogen). After heat inactivation (65°C, 15 min), the DNA was digested with 5 u of AvaII (NEB) for 30 min at 37°C. Linkers (a mixture of 30 pmol of each A and T; supplementary Table 1) were ligated using 1 U of T4 ligase (Invitrogen). Following overnight incubation at 18°C, an additional 5 U of AvaII and 0.5 U of T4 ligase were added and the mixture was further incubated for 30 min at 37°C. The DNA was purified on ERC MinElute columns (Qiagen) and subjected to 45 cycles of PCR amplifications with *Taq* polymerase (Invitrogen) and primers described in supplementary Table 1. PCR products were resolved by 6% PAGE and stained with ethidium bromide. Bands of interest were excised and the DNA was 'freeze and squeezed' using SpinX columns (Corning). The DNA from each band was PCR-reamplified and digested with each of MspI and HinfI (NEB). Following 3.5% agarose gel electrophoresis, bands of interest were excised and the DNA was eluted using Qiagen gel extraction kit and sequenced on ABI 3100 Genetic Analyzer using BigDye kit (ABI). Sequences were mapped onto the mouse genome (Assembly m36) using the SSAHA program on the Ensembl web server (www.ensembl.org).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described (20) with the oligonucleotides shown in Supplementary Table 1.

Plasmids and transfections

The −751/+12 and +265/+907 *Osbpl8* gene fragments were PCR-amplified from MC3T3-E1 genomic DNA using the primers shown in supplementary Table 1 and Pfx polymerase (Invitrogen). The amplicons were inserted, respectively, between the HindIII and the BglII sites of pGL3 basic (Promega), or, between the HindIII and the BamHI sites of TK-luc, in which luciferase is driven by the thymidine kinase promoter. Plasmids were confirmed by restriction digestion and partial sequencing. ST2 cells were transfected and luciferase activity was measured two days later as previously described (20).

RT–PCR

RNA was prepared using TRIZOL reagent (Invitrogen) and treated with DNaseI (Ambion). Reverse transcription was performed with Thermoscript polymerase (Invitrogen) using oligo(dT) for priming. PCR was performed with *Taq* polymerase (Invitrogen) and the primers described in supplementary Table 1 in the presence of [α-^{32}P]dCTP (20). PCR products were subjected to PAGE and visualized using Storm 840 phosphoimager (Molecular Dynamics).

RESULTS

ChIP Display

The main problem in cloning targets for a transcription factor directly from immunoprecipitated chromatin is the overall abundance of non-specifically co-precipitated DNA: although fragments containing each given target site can be 10- to 30-fold more abundant than fragments representing any random locus, the non-specifically precipitated fragments altogether overwhelm the specific ones (Figure 1A). To overcome this obstacle we devised a method, ChIP Display, which effectively concentrates fragments containing each target while scattering the remaining DNA. We concentrate targets via restriction digestion: fragments containing a certain target site now have the same size, allowing us to concentrate them on a gel. Scattering of the non-specifically precipitated fragments is achieved by dividing the total pool of restriction fragments into families based on the identity of nucleotides at the ends of such fragments (highlighted in Figure 2). Since all restriction fragments containing each given target have the same nucleotides at the ends, they remain in the same family and the signal is not eroded. In contrast, the other fragments, mostly background, are scattered into many families.

The CD protocol is rather simple (Figure 1). Following ChIP, immunoprecipitated DNA is dephosphorylated by shrimp alkaline phosphatase (SAP) to prevent ligation of linkers to DNA ends generated by sonication (Figure 1B). SAP is then heat-inactivated and the DNA is digested with a restriction enzyme. We used AvaII, whose recognition sequence, GGWCC (W = A or T), can be expected approximately every 500 bp in a random sequence. Linkers are then ligated, which do not restore the AvaII sites (Figure 2A; Supplementary

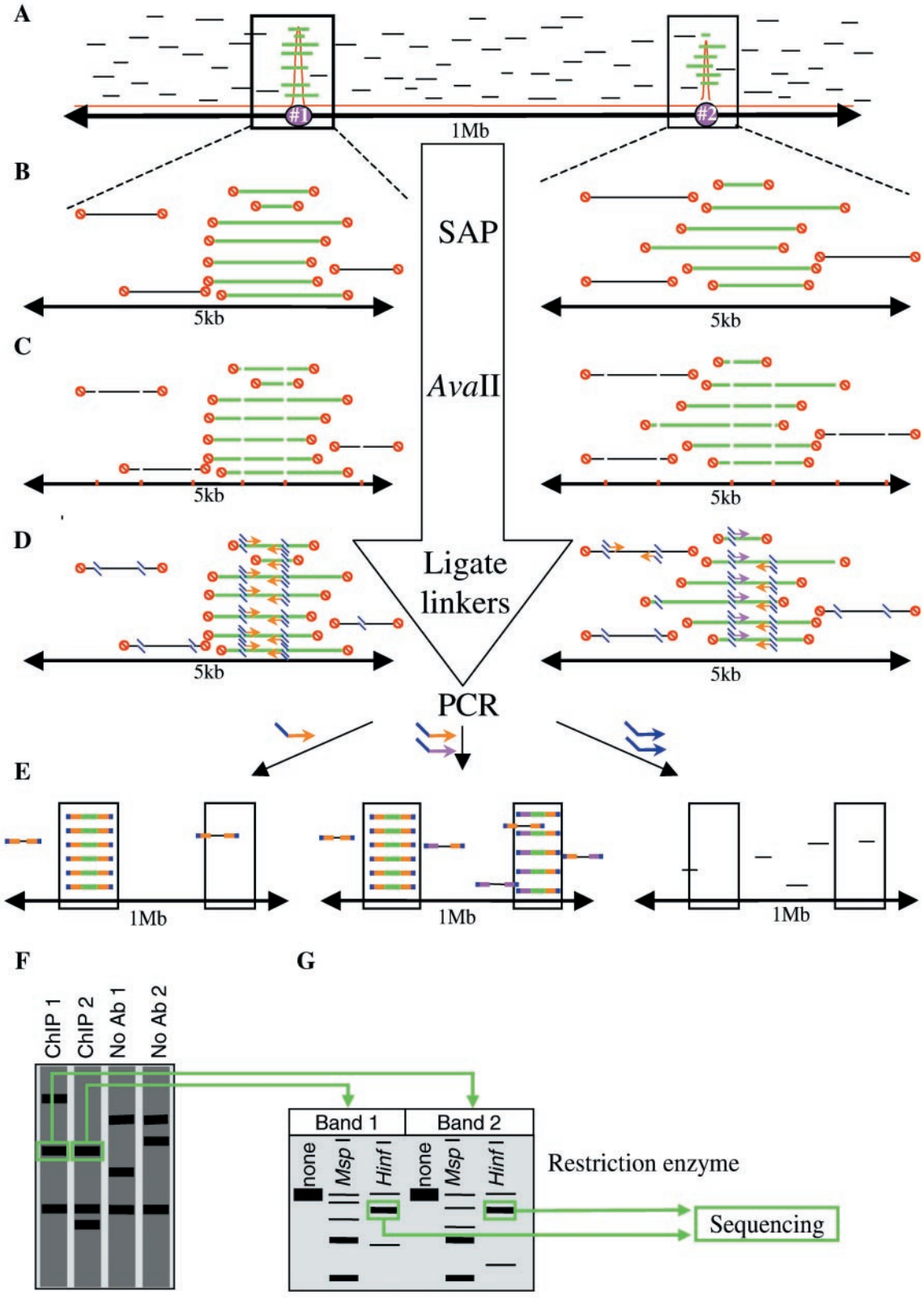

Figure 1. Principles of ChIP Display. (**A**) Precipitated DNA fragments (green—specific; black—non-specific) are aligned with the genome. Graph (red) shows the representation of each nucleotide in the immunoprecipitate. The area shown contains two hypothetical target genes (#1 and #2). (**B**) Magnification of the two regions of interest from (A). DNA fragments are treated with shrimp alkaline phosphatase (SAP) to prevent linker ligation to DNA ends generated during sonication. Red circles, dephosphrylated end. (**C**) DNA is digested with AvaII. (**D**) Linkers are ligated to the ends of AvaII fragments. Also shown are the nested primers that can amplify targets #1 and #2. (**E**) PCR products amplified in three reactions: left—target #1 is amplified in a reaction with a single primer (family *X* in Figure 2B); middle—target #2 is amplified with two different primers (family *V* in Figure 2B); note that target #1 is amplified here again; right—most of the PCR reactions will amplify neither target #1 nor target #2. (**F**) Schematic illustration of PAGE of PCR products from two ChIPs and two mock ChIPs (*no Ab*) belonging to one *CD* family. (**G**) Each of the two co-migrating bands from (F) is excised, reamplified and restriction-analyzed with four cutters. Major co-migrating restriction products are then isolated and directly sequenced.

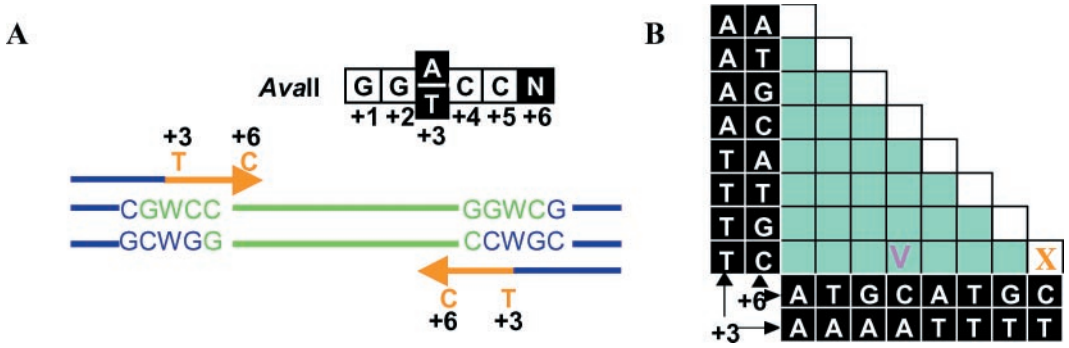

Figure 2. CD families. (**A**) Design of linkers and nested primers. Positions +1 to +6 are defined at the top. Nucleotides at positions +3 and +6 (highlighted) were used to segregate AvaII fragments into families. Linkers contain a C at position +1 to destroy the AvaII sites and a 1:1 A:T mixture (*W*) at position +3. In addition, this panel shows one of the eight PCR nested primers, the one with T at position +3 and C at position +6. (**B**) The eight nested primers produce 36 combinations for PCR. White square—a single primer. Shaded square—two different primers.

Table 1), so that ligation can be performed in the presence of AvaII to recycle fragments that ligate to each other. Thirty-six combinations of eight nested primers are then employed to amplify fragments belonging to one family at a time (Figure 2B). Each such primer contains either A or T at the +3 position of the destroyed AvaII site and one nested nucleotide, A, T, G or C, at the 3′end (Figure 2). Amplified fragments from two to three independent immunoprecipitates and two to three no antibody control precipitates are resolved by PAGE (Figure 1F). Bands reproduced in all the ChIP lanes and in none of the control lanes are considered candidate targets and are excised from the gel for further characterization.

Identification of novel RUNX2 targets

We are using CD in osteoblasts to discover novel transcription targets for RUNX2, a DNA-binding protein that plays a pivotal role in bone and cartilage development (5,21), but whose direct targets are largely unknown. RUNX2 ChIP was performed in day 8 MC3T3-E1 osteoblastic cultures, in which we have previously demonstrated RUNX2 interaction with the *osteocalcin* promoter (20). Figure 3A presents PAGE of the PCR products belonging to a CD family, the one marked by *X* in Figure 2B. A band of ∼350 bp is present in each lane derived from a RUNX2 ChIP but not in any lane representing a mock (no antibody) ChIP. This band was excised from each ChIP lane, eluted from the gel and reamplified for identification.

Direct sequencing of the eluted material is not feasible because of contamination with co-migrating non-specific fragments. Moreover, sequencing with a primer that anneals to the linker is impossible since the same linkers are present at both ends. We take an approach that again can be described as 'scattering'. The reamplified material from each band is digested by a set of four cutters and the products are resolved on an agarose gel (Figures 1G and 3B). The patterns obtained from each CD are compared and restriction fragments of similar size are excised and now directly sequenced with a linker-primer. This restriction digestion step serves several purposes: (i) it verifies that co-migrating bands from different ChIPs indeed represent the same genomic fragment; (ii) it separates the band of interest from background DNA that can interfere with sequencing; and (iii) sub-fragments no longer have the

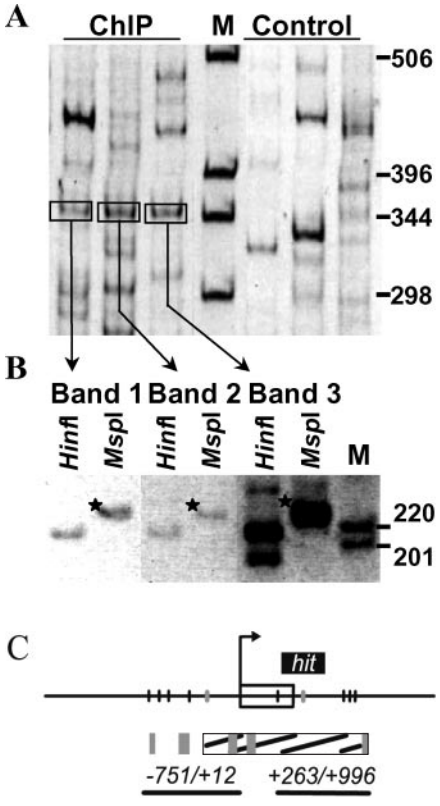

Figure 3. CD identifies a novel RUNX2 target gene. (**A**) Three independent RUNX2 chromatin immunoprecipitates (ChIP) and three no-antibody *controls* were subjected to CD procedure using a single nested primer (with T and C at positions +3 and +6, respectively; see Figure 1). Amplification products were resolved in 6% polyacrylamide gel and visualized by ethidium bromide staining. The three bands common to all three ChIPs (boxed) were excised. (**B**) The DNA eluted from each of the bands in (A) was reamplified, digested with either HinfI or MspI, and subjected to gel electrophoresis. Major restriction products were sequenced and all mapped to the vicinity of the *Osbpl8* start site. (**C**) Schematic illustration of a 3 kb genomic region (horizontal line) centered around the *Osbpl8* transcription start site. The first exon is represented by the white box. Black box ('hit') denotes the AvaII fragment that was amplified during the *CD* procedure. The hit is located inside a CpG island (striped box) but does not overlap with repetitive sequences (gray boxes). Ten putative RUNX2 binding sites are shown at the top as vertical bars perpendicular to the horizontal line. The darker bars represent those RUNX2 sites that were confirmed by EMSA (Figure 3A). The −751/+12 and +263/+996 fragments, which were cloned into reporter vectors, are shown at the bottom.

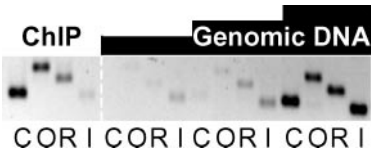

Figure 4. Validation of CD hits. Association of RUNX2 with the *Osbpl8* gene (O), *as well as the Runx3* gene (R), was tested using conventional ChIP assay. Fragments of *Osbpl8* (O) *and Runx3* (R) genes were PCR amplified with promoter-specific primers listed in Supplementary Table 1. Collagen alpha1(I) (8) (C) and insulin (I) were used as positive and negative controls, respectively. Genomic DNA was used to demonstrate dynamic range of PCR amplification.

same ends and can thus be sequenced directly. Sequences obtained in this manner from the three bands indicated in Figure 3B by an asterisk, were aligned with the mouse genome using the SSAHA program on the ENSEMBL website (www.ensembl.org) and all mapped to the first exon–intron boundary of the gene encoding Oxysterol Binding Protein-Like 8 (OSBPL8) (22). The location of the hit in the wide context of the *Osbpl8* locus is shown in Figure 3C and supplementary Figure 1. OSBPL8, a.k.a. Oxysterol binding protein Related Protein 8 (ORP8), has an oxysterol-binding domain, a bipartite nuclear localization signal and a pleckstrin homology domain (www.ensembl.org). Interestingly, oxysterols are secreted by and promote osteoblast differentiation and mineralization (23–25).

Additional novel RUNX2 targets identified by CD of two other families (Supplementary Figure 1) encode (i) RUNX3, another member of the RUNX family of transcription factors, which plays a role in gut differentiation; although RUNX2 is the RUNX member with the most well-established role in osteoblast differentiation and bone formation (26), the other RUNX proteins, RUNX1 and RUNX3, may also play a role in this process (27,28); (ii) FLI1, a member of the Ets family of transcription factors, which plays a role in hematopoiesis (29) and is also expressed in osteoblasts (30); and (iii) a novel EST (accession no. BI964607), encoding a protein homologuous to the DYRK1A kinase (31). In all of these cases, the CD hit was mapped to a location within 1 kb of the transcription start site.

For each of the four RUNX2 CD hits, we confirmed the following: (i) sequences obtained from co-migrating bands from independent ChIPs were identical; (ii) the observed size and internal restriction sites for the AvaII fragment were consistent with the database sequence; (iii) the AvaII fragment was amplifiable with the specific primers used for CD; and, most importantly, (iv) interaction of RUNX2 with each target was confirmed by conventional ChIP assay with gene-specific primers (Figure 4 and Supplementary Figure 2).

Osbpl8 gene is regulated by RUNX2

We decided to further study the regulation of the *Osbpl8* gene by RUNX2. Sequence analysis (TFSEARCH program, Dr Y. Akiyama, www.rwcp.or.jp/papia/) revealed the presence of ten putative RUNX sites in the vicinity of the transcription start. Five are located within 5′ flanking sequences, one in the first exon and four in the first intron (Figure 3C). In EMSA using MC3T3-E1 cell extract as a source of RUNX2, three of these sites (at positions −667, −405 and +276) bound RUNX2 at least as strongly as the classical RUNX element from the osteocalcin promoter [OSE2; (32)], 5 sites (−711, −559, +801, +851, +892) bound RUNX2 more weakly and 2 were incapable of binding (Figure 5A). Identity of the complexes was confirmed by supershift assay and competition with the OSE2 element from the *osteocalcin* promoter. Furthermore, in transient transfection assays of ST2 bone marrow stroma-derived pre-osteoblast cultures, co-transfection of RUNX2 stimulated by 1.6-fold the transcription from a plasmid, in which the +263/+996 *Osbpl8* intronic fragment (Figure 3C) was fused upstream of the heterologous TK promoter (Figure 5B). In contrast, a 1.5-fold repression was observed with AML1-ETO (33,34), a dominant-negative RUNX protein. These effects were similar to those observed with the basal *osteocalcin* gene promoter, a classical RUNX2 target (32) (Figure 5B). The −751/+12 *Osbpl8* promoter fragment was also repressed by AML1-ETO, although no activation was observed with wild-type RUNX2. Altogether, the ChIP, EMSA and transfection assays suggest that *Osbpl8* is a target gene for RUNX2 and could therefore be differentially regulated during osteoblast differentiation.

Osbpl8 gene expression was measured during BMP-2-induced osteoblast differentiation of the ST2 cell cultures (35). *Osteoclacin*, a classical marker of osteoblast differentiation and a target of RUNX2 (13,20,32), was used as control. As shown in Figure 5C, both osteoclacin and *Osbpl8* mRNA strongly increased as a function of time in response to BMP-2 treatment. These results demonstrate the functional consequences of RUNX2 binding to the endogenous *Osbpl8* locus.

DISCUSSION

ChIP Display, a method for the identification of novel transcription factor targets in higher eukaryotes, is significantly different from other reported ChIP-based approaches (2–4). Bigler and Eisenman constructed a DNA library of about 1000 clones from chromatin immunoprecipitated with antibodies against the thyroid hormone receptor (TR) and identified 2 potential TR targets by screening for TR binding *in vitro* (3,36). However since non-specifically precipitated DNA fragments can fortuitously bind the protein *in vitro* (but not *in vivo*) utility of this approach is questionable. Weinmann *et al*. (37) improved the efficiency of ChIP cloning by performing double ChIP and identified eight E2F1 and E2F4 targets. In addition to difficulties inherent to double ChIP, a large number of false positives was still reported and the authors suggested that application of the method was limited (2). CD can disclose transcription targets in ChIPs that are enriched for targets by as little as 10 to 20-fold. Despite this limited enrichment, the four hits obtained in our initial CD experiments were all confirmed as targets using conventional ChIP assays (Figure 4 and Supplementary Figure 2).

Several investigators have hybridized immunopecipitated DNA to genomic microarrays for the identification of targets. While this approach is ideal for yeast (38,39), its application to higher eukaryotes is significantly hindered by the size of their genomes. Ren *et al*. (40) hybridized E2F1 and E2F4 ChIPs to microarrays containing the −700 to +200 promoter regions of 1500 human genes selected based on their known cell cycle dependency. Such arrays are not only biased towards the handpicked genes, but would also fail to identify *cis*-acting

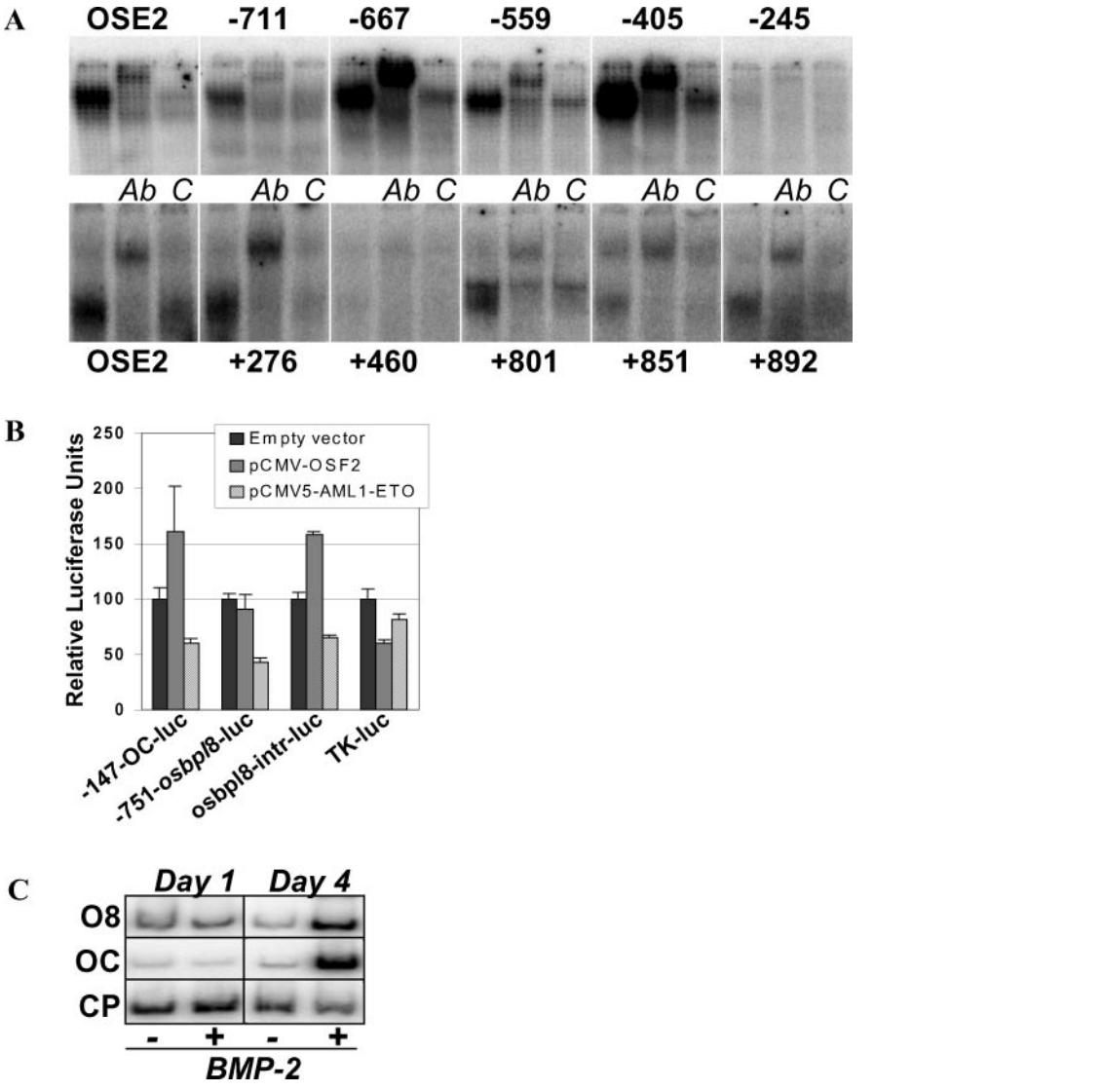

Figure 5. *Osbpl8* expression is regulated by RUNX2. (**A**) *Osbpl8* transcription start site is surrounded by RUNX2-binding elements. MC3T3-E1 cell extract was subjected to EMSA with 20 bp oligonucleotide probes centered around putative RUNX2 binding sites located at the given positions relative to the *Osbpl8* transcription start site. Positive control was the osteoblast-specific element 2 probe (*OSE2*) from the osteocalcin gene promoter (32). The RUNX2 complex was either competed (*C*) with unlabeled OSE2 oligonucleotide or supershifted with anti RUNX2 antibodies (*Ab*). (**B**) *Osbpl8* gene regulatory sequences surrounding the transcription start site confer RUNX2 responsiveness. ST2 marrow stroma-derived cells were transiently transfected with 3 μg of the indicated reporter plasmid and 0.5 μg of the indicated expression vector. Reporter plasmid containing the osteocalcin promoter, a classical RUNX2 target, was used as a positive control (32). Luciferase activity with the empty vector was set at 100 (raw values were 26, 2532, 748 and 838 for -147-OC-luc, -751-osbpl8-luc, osbpl8-intr-luc and TK-luc, respectively). Data are Mean ± SEM ($n = 3$). (**C**) *Osbpl8* gene expression parallels development of the osteoblast phenotype. ST-2 cells were treated at confluency (day 0) with 300 ng/ml BMP2 to induce osteoblast differentiation. RNA was extracted on the indicated days for RT–PCR analysis of Osbpl8 (O8), osteocalcin (OC) and cyclophylin (CP) mRNAs.

elements distant from transcription start sites. Indeed, recent analysis of Sp1 binding to human chromosomes 21 and 22 showed that only 27% of the occupied sites were located within 1 kb of the 5′ exon (4). In another study, Weinmann *et al*. used microarrays containing 8000 CpG islands, which are often associated with gene promoters (41,42). That CpG islands represent only a fraction of genomic sequences to which transcription factors bind is well demonstrated both by the experiments of Cawley *et al*. (4) with Sp1, c–myc and p53 and by our initial CD analysis with RUNX2: of the four RUNX2 targets identified so far, only one, *Osbpl8*, overlaps with a CpG island (Figure 3C and Supplementary Figure 1).

Hybridization of ChIP to microarrays would be free of bias if the entire genome could be represented on those arrays. Cawley *et al*. (4) used oligonucleotide microarrays representing most of human chromosomes 21 and 22 for comprehensive analysis of the genomic sites occupied by the transcription factors Sp1, c-myc and p53. Limitations of this study include the coverage of only two chromosomes and the high cost. In the absence of a whole genome comprehensive microarray, and given the anticipated limited access to such technology,

CD will be useful for the identification of targets for transcription factors, in mammalian cells.

In conclusion, ChIP Display facilitates identification of direct binding targets for transcription factors. Depending on the crosslinking reagent used for ChIP, CD can also be used for the discovery of genomic targets for proteins that associate with DNA indirectly. CD is not suited for comprehensive analysis of binding sites for proteins that interact with a large number of genomic sites (e.g. SP1, DNA polymerase II or HDACs). It is probably more suited to identify targets for transcription factors with more limited number of targets. CD does not require expensive technologies, but can be performed with basic molecular biology equipment. It is relatively insensitive to the high background typically associated with ChIP. Genomic sites more highly occupied by the protein of interest will yield stronger signal. Furthermore, CD gels allow visual comparison among targetomes of transcription factor(s) in various cell types and/or under different physiological conditions and identification of only those bands that represent *in vivo* protein–DNA interactions of biologically attractive nature, such as cell-type-specific or hormone-responsive gene occupancy. For example, different targets of RUNX2 in osteoblasts versus chondrocytes could be disclosed by displaying side-by-side the respective CD families from the two cell types, followed by the isolation and sequencing of only those bands that are specific to either osteoblasts or chondrocytes.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Abel Valdovinos for technical assistance, Nathalie Leclerc, Tommy Noh, Steven Pregizer and Elisheva Smith for suggestions, Gerhard Coetzee and Joseph Hacia for critically reading the maniscript, Wyeth Research for the generous gift of recombinant human BMP-2, Gerard Karsenty for plasmids and the National Institutes of Health for funding (AR49297 and AR47052).

REFERENCES

1. Liang,P. and Pardee,A.B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, **257**, 967–971.
2. Weinmann,A.S. and Farnham,P.J. (2002) Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. *Methods*, **26**, 37–47.
3. Bigler,J. and Eisenman,R.N. (1994) Isolation of a thyroid hormone-responsive gene by immunoprecipitation of thyroid hormone receptor–DNA complexes. *Mol. Cell. Biol.*, **14**, 7621–7632.
4. Cawley,S., Bekiranov,S., Ng,H.H., Kapranov,P., Sekinger,E.A., Kampa,D., Piccolboni,A., Sementchenko,V., Cheng,J., Williams,A.J. *et al.* (2004) Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell*, **116**, 499–509.
5. Komori,T., Yagi,H., Nomura,S., Yamaguchi,A., Sasaki,K., Deguchi,K., Shimizu,Y., Bronson,R.T., Gao,Y.H., Inada,M. *et al.* (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, **89**, 755–764.
6. Ducy,P., Zhang,R., Geoffroy,V., Ridall,A.L. and Karsenty,G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*, **89**, 747–754.
7. Lee,M.H., Javed,A., Kim,H.J., Shin,H.I., Gutierrez,S., Choi,J.Y., Rosen,V., Stein,J.L., van Wijnen,A.J., Stein,G.S. *et al.* (1999) Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. *J. Cell. Biochem.*, **73**, 114–125.
8. Kern,B., Shen,J., Starbuck,M. and Karsenty,G. (2001) Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. *J. Biol. Chem.*, **276**, 7101–7107.
9. Sato,M., Morii,E., Komori,T., Kawahata,H., Sugimoto,M., Terai,K., Shimizu,H., Yasui,T., Ogihara,H., Yasui,N. *et al.* (1998) Transcriptional regulation of osteopontin gene *in vivo* by PEBP2alphaA/CBFA1 and ETS1 in the skeletal tissues. *Oncogene*, **17**, 1517–1525.
10. Kundu,M., Javed,A., Jeon,J.P., Horner,A., Shum,L., Eckhaus,M., Muenke,M., Lian,J.B., Yang,Y., Nuckolls,G.H. *et al.* (2002) Cbfbeta interacts with Runx2 and has a critical role in bone development. *Nature Genet.*, **32**, 639–644.
11. Sierra,J., Villagra,A., Paredes,R., Cruzat,F., Gutierrez,S., Javed,A., Arriagada,G., Olate,J., Imschenetzky,M., Van Wijnen,A.J. *et al.* (2003) Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D3 receptor but not p300 intrinsic histone acetyltransferase activity. *Mol. Cell. Biol.*, **23**, 3339–3351.
12. Westendorf,J.J., Zaidi,S.K., Cascino,J.E., Kahler,R., van Wijnen,A.J., Lian,J.B., Yoshida,M., Stein,G.S. and Li,X. (2002) Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. *Mol. Cell. Biol.*, **22**, 7982–7992.
13. Thomas,D.M., Carty,S.A., Piscopo,D.M., Lee,J.S., Wang,W.F., Forrester,W.C. and Hinds,P.W. (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol. Cell*, **8**, 303–316.
14. D'Alonzo,R.C., Selvamurugan,N., Karsenty,G. and Partridge,N.C. (2002) Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for collagenase-3 promoter activation. *J. Biol. Chem.*, **277**, 816–822.
15. Kim,S., Koga,T., Isobe,M., Kern,B.E., Yokochi,T., Chin,Y.E., Karsenty,G., Taniguchi,T. and Takayanagi,H. (2003) Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. *Genes Dev.*, **17**, 1979–1991.
16. Kahler,R.A. and Westendorf,J.J. (2003) Lymphoid enhancer factor-1 and beta-catenin inhibit Runx2-dependent transcriptional activation of the osteocalcin promoter. *J. Biol. Chem.*, **278**, 11937–11944.
17. Mundlos,S., Otto,F., Mundlos,C., Mulliken,J.B., Aylsworth,A.S., Albright,S., Lindhout,D., Cole,W.G., Henn,W., Knoll,J.H. *et al.* (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*, **89**, 773–779.
18. Otto,F., Thornell,A.P., Crompton,T., Denzel,A., Gilmour,K.C., Rosewell,I.R., Stamp,G.W., Beddington,R.S., Mundlos,S., Olsen,B.R. *et al.* (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, **89**, 765–771.
19. Smith,E., Redman,R.A., Logg,C.R., Coetzee,G.A., Kasahara,N. and Frenkel,B. (2000) Glucocorticoids inhibit developmental stage-specific osteoblast cell cycle. Dissociation of cyclin A-cyclin-dependent kinase 2 from E2F4-p130 complexes. *J. Biol. Chem.*, **275**, 19992–20001.
20. Luppen,C.A., Leclerc,N., Noh,T., Barski,A., Khokhar,A., Boskey,A.L., Smith,E. and Frenkel,B. (2003) Brief bone morphogenetic protein 2 treatment of glucocorticoid-inhibited MC3T3-E1 osteoblasts rescues commitment-associated cell cycle and mineralization without alteration of Runx2. *J. Biol. Chem.*, **278**, 44995–45003.
21. Enomoto,H., Furuichi,T., Zanma,A., Yamana,K., Yoshida,C., Sumitani,S., Yamamoto,H., Enomoto-Iwamoto,M., Iwamoto,M. and Komori,T. (2004) Runx2 deficiency in chondrocytes causes adipogenic changes *in vitro*. *J. Cell. Sci.*, **117**, 417–425.
22. Anniss,A.M., Apostolopoulos,J., Dworkin,S., Purton,L.E. and Sparrow,R.L. (2002) An oxysterol-binding protein family identified in the mouse. *DNA Cell. Biol.*, **21**, 571–580.
23. Silva,J., Beckedorf,A. and Bieberich,E. (2003) Osteoblast-derived oxysterol is a migration-inducing factor for human breast cancer cells. *J. Biol. Chem.*, **278**, 25376–25385.
24. Watson,K.E., Bostrom,K., Ravindranath,R., Lam,T., Norton,B. and Demer,L.L. (1994) TGF-beta 1 and 25-hydroxycholesterol

stimulate osteoblast-like vascular cells to calcify. *J. Clin. Invest.*, **93**, 2106–2113.
25. Kha,H.T., Basseri,B., Shouhed,D., Richardson,J., Tetradis,S., Hahn,T.J. and Parhami,F. (2004) Oxysterols regulate differentiation of mesenchymal stem cells: pro-bone and anti-fat. *J. Bone Miner. Res.*, **19**, 830–840.
26. Banerjee,C., McCabe,L.R., Choi,J.Y., Hiebert,S.W., Stein,J.L., Stein,G.S. and Lian,J.B. (1997) Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J. Cell. Biochem.*, **66**, 1–8.
27. Lian,J.B., Balint,E., Javed,A., Drissi,H., Vitti,R., Quinlan,E.J., Zhang,L., Van Wijnen,A.J., Stein,J.L., Speck,N. *et al.* (2003) Runx1/AML1 hematopoietic transcription factor contributes to skeletal development *in vivo*. *J. Cell. Physiol.*, **196**, 301–311.
28. Yamashiro,T., Aberg,T., Levanon,D., Groner,Y. and Thesleff,I. (2002) Expression of Runx1, -2 and -3 during tooth, palate and craniofacial bone development. *Mech. Dev.*, **119** (Suppl 1), S107–S110.
29. Truong,A.H. and Ben-David,Y. (2000) The role of Fli-1 in normal cell function and malignant transformation. *Oncogene*, **19**, 6482–6489.
30. Vary,C.P., Li,V., Raouf,A., Kitching,R., Kola,I., Franceschi,C., Venanzoni,M. and Seth,A. (2000) Involvement of Ets transcription factors and targets in osteoblast differentiation and matrix mineralization. *Exp. Cell. Res.*, **257**, 213–222.
31. Becker,W., Weber,Y., Wetzel,K., Eirmbter,K., Tejedor,F.J. and Joost,H.G. (1998) Sequence characteristics, subcellular localization, and substrate specificity of DYRK-related kinases, a novel family of dual specificity protein kinases. *J. Biol. Chem.*, **273**, 25893–25902.
32. Ducy,P. and Karsenty,G. (1995) Two distinct osteoblast-specific *cis*-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.*, **15**, 1858–1869.
33. Meyers,S., Downing,J.R. and Hiebert,S.W. (1993) Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein–protein interactions. *Mol. Cell. Biol.*, **13**, 6336–6345.
34. Meyers,S., Lenny,N. and Hiebert,S.W. (1995) The t(8;21) fusion protein interferes with AML-1B-dependent transcriptional activation. *Mol. Cell. Biol.*, **15**, 1974–1982.
35. Yamaguchi,A., Ishizuya,T., Kintou,N., Wada,Y., Katagiri,T., Wozney,J.M., Rosen,V. and Yoshiki,S. (1996) Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. *Biochem. Biophys. Res. Commun.*, **220**, 366–371.
36. Bigler,J. and Eisenman,R.N. (1995) Novel location and function of a thyroid hormone response element. *EMBO J.*, **14**, 5710–5723.
37. Weinmann,A.S., Bartley,S.M., Zhang,T., Zhang,M.Q. and Farnham,P.J. (2001) Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol. Cell. Biol.*, **21**, 6820–6832.
38. Ren,B., Robert,F., Wyrick,J.J., Aparicio,O., Jennings,E.G., Simon,I., Zeitlinger,J., Schreiber,J., Hannett,N., Kanin,E. *et al.* (2000) Genome-wide location and function of DNA binding proteins. *Science*, **290**, 2306–2309.
39. Kurdistani,S.K., Robyr,D., Tavazoie,S. and Grunstein,M. (2002) Genome-wide binding map of the histone deacetylase Rpd3 in yeast. *Nature Genet.*, **31**, 248–254.
40. Ren,B., Cam,H., Takahashi,Y., Volkert,T., Terragni,J., Young,R.A. and Dynlacht,B.D. (2002) E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev.*, **16**, 245–256.
41. Weinmann,A.S., Yan,P.S., Oberley,M.J., Huang,T.H. and Farnham,P.J. (2002) Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. *Genes Dev.*, **16**, 235–244.
42. Wells,J., Yan,P.S., Cechvala,M., Huang,T. and Farnham,P.J. (2003) Identification of novel pRb binding sites using CpG microarrays suggests that E2F recruits pRb to specific genomic sites during S phase. *Oncogene*, **22**, 1445–1460.