

Use of altered-specificity binding Oct-4 suggests an absence of pluripotent cell-specific cofactor usage

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Received June 9, 2005; Revised and Accepted September 29, 2005

ABSTRACT

Oct-4 is a POU domain transcription factor that is critical for maintaining pluripotency and for stem cell renewal. Previous studies suggest that transcription regulation by Oct-4 at particular enhancers requires the input of a postulated E1A-like cofactor that is specific to pluripotent cells. However, such studies have been limited to the use of enhancer elements that bind other POU-protein family members in addition to Oct-4, thus preventing a 'clean' assessment of any Oct-4:cofactor relationships. Other attempts to study Oct-4 functionality in a more 'stand-alone' situation target Oct-4 transactivation domains to DNA using heterologous binding domains, a methodology which is known to generate artificial data. To circumvent these issues, an altered-specificity binding Oct-4 (Oct-4RR) and accompanying binding site, which binds Oct-4RR only, were generated. This strategy has previously been shown to maintain Oct-1:cofactor interactions that are highly binding-site and protein/binding conformation specific. This system therefore allows a stand-alone study of Oct-4 function in pluripotent versus differentiated cells, without interference from endogenous POU factors and with minimal deviation from bound wild-type protein characteristics. Subsequently, it was demonstrated that Oct-4RR and the highly transactive regions of its N-terminus determined here, and its C-terminus, have the same transactivation profile in pluripotent and differentiated cells, thus providing strong evidence against the existence of such a pluripotent cell-specific Oct-4 cofactor.

INTRODUCTION

Mouse Oct-4 is a member of the POU domain transcription factor family with an expression pattern that is restricted to pluripotent and totipotent cells of the animal and embryonic stem (ES) and carcinoma (EC) cell lines (1–3). Treatment of the pluripotent P19 EC cell line with the retinoic acid (RA) morphogen stimulates cell differentiation with a parallel downregulation in Oct-4 expression (1). (For the purposes of simplicity, cell lines which are not generally considered pluripotent, such as P19 cells which have been treated with RA, or HeLa and NIH3T3 cells, shall henceforth be referred to as differentiated cells). Oct-4 is critical for the maintenance of cell pluripotency and for stem cell renewal. P19 cells differentiated by fusion to fibroblast cells can undergo de-differentiation in response to ectopic Oct-4 expression, while exogenous expression of Oct-4 in Oct-4-knockout cells is also sufficient to rescue pluripotency (4,5). Conversely, embryos in which Oct-4 expression is knocked out fail to maintain pluripotency within the inner cell mass (6).

A widely studied system for examining Oct-4 function is the 6W enhancer element, which is based on an immunoglobulin heavy chain (IgH) enhancer and contains multimerized Oct-protein binding sites (the consensus octamer motif is ATGCAAAT), as well as a β helix–loop–helix (β HHLH) binding motif. This element is associated with a high level of downstream reporter gene transactivation in pluripotent EC cells which ordinarily express Oct-4, such as undifferentiated P19 (P19U) cells (7,8), but importantly not in non-Oct-4 expressing, differentiated cell lines, when Oct-4 is expressed exogenously (5–9). As 6W activity is dependent on functional octamer motif (8) and as Oct-4 is the only known POU transcription factor specific to undifferentiated P19 cells, Oct-4 has been proposed as a likely candidate for involvement in undifferentiated cell-specific 6W activity. This suggestion has been strengthened by the observation that antisense Oct-4, but not antisense Oct-1, results in a relative drop in the activity

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of octamer motif-containing enhancer DNA in P19U cells (1). Taken together, the above observations suggest the existence of a cell-specific transcription cofactor, present only in undifferentiated cells, which might also specifically interact with Oct-4.

While attempts to identify such a cell-specific cofactor have not yet been successful, a few interesting observations have been made which provide some evidence for an Oct-4:cofactor paradigm. For example, Oct-4 has been shown to interact with the adenovirus protein E1A, resulting in transcriptional upregulation from 'distal' 6W enhancer positions. Furthermore, an E1A-like protein is thought to exist in undifferentiated P19 cells, although again it has not yet been identified (8–10). It is perhaps therefore not surprising that the hypothesized E1A-like factor and postulated Oct-4 cofactor are often suggested to be the same thing. The relevance of any Oct-4:E1A relationship is unclear however, as E1A has been shown to interact with DNA in a non-specific manner and is thought to be able to directly regulate transcription through the TATA box and TBP or TAFs as well as numerous additional transcription factors and cofactors [reviewed in (11,12)].

The observation that the papillomavirus protein E7 can also function as an Oct-4 transcription cofactor may further suggest a common Oct-4 cofactor paradigm (9). Indeed, others have drawn on a number of Oct-4 studies as well as their own work to propose a theoretical model in which a certain subset of Oct-4 genes, which are 'squashed' by over-expression of Oct-4, require a cofactor(s) which in circumstances of high nuclear Oct-4 concentrations may be sequestered away from the Oct-4:DNA complex (13,14). However, this cofactor model is only suggestive of the existence of Oct-4 cofactors and not that such factors are particular to pluripotent cells and an Oct-4 partnership.

Studies of Oct-4 functionality in pluripotent cells to date have focused on the use of Oct-4 activation domains fused to heterologous binding domains, such as the GAL4 DNA-binding domain, or through an endogenous Oct-4-knockout system for which complementation by various Oct-4 mutants allows determination of functional domains (5,15). Such systems have demonstrated that both Oct-4 N- and C-terminus activation domains contribute to Oct-4 functionality. However, fusion proteins comprising heterologous DNA binding motifs, although representing the only effective route for the analysis of cell-specific activity without contributions from the endogenous protein, are known to generate data that are not representative of true wild-type protein function. This therefore suggests that the Oct-4 DNA-binding domain contributes to functional activity and that intra protein structure or a particular Oct-4 binding configuration is essential for correct transcription regulation by Oct-4 (15).

An alternative and more elegant route is that employed by Pomerantz and Sharp (16), who showed that by changing amino acids Valine-47 and Asparagine-51 within the POU homeodomain of Oct-1 to arginine residues, the protein (Oct-1RR) would bind a mutated octamer element ATGCAAGG with a significantly higher efficiency than the wild-type protein. Importantly, the Oct-1RR protein retains the ability to interact with the Herpes simplex virus coactivator VP16 and the cellular protein Host cell factor, in a manner analogous to that of wild-type protein, which has been shown to be highly dependant on the conformation of

promoter-bound Oct-1 (17). Other studies have shown that both Oct-1RR and Oct-2RR can functionally replace Oct-1/2 in the interaction with the B-cell specific coactivator Bob-1, again confirming the structural and functional integrity of the modified Oct-protein–DNA complex (18). Translating this altered-specificity binding strategy to Oct-4 would therefore maintain any wild-type protein/binding configuration that could be essential for proper protein function, while eliminating any endogenous protein contributions in functional assays.

Here, we employ an altered-specificity system incorporating the double amino acid substitution Oct-4 (Oct4RR), which alters Oct-4 DNA binding specificity to the sequence 5'-ATGCAAGG-3'. We demonstrate that this sequence is not bound efficiently by wild-type Oct-4 or other POU domain proteins, thus eliminating the contribution of endogenous proteins in functional assays to enable a 'stand-alone' assessment of Oct-4 transcriptional regulation and potential use of cofactors. Data presented here show that Oct-4RR has an equivalent transactivation profile in pluripotent and differentiated cells, thus challenging the current hypothesis that Oct-4 utilizes a pluripotent cell-specific cofactor on such regulatory elements. Activation domain deletions in concert with altered-specificity binding mutants were also constructed and used to assess any cell-specific requirement for particular Oct-4 subdomains. Oct-4-mediated transactivation was also shown to be predominantly a function of the N-terminus activation domain, with the C-terminus activation domain displaying a lower but still significant transactivation function, again with neither activation domain displaying any cell specificity.

MATERIALS AND METHODS

General reagents and chemicals

General chemicals were supplied by Sigma Aldrich, Invitrogen, ICN, VWR, Amersham Pharmacia or Scientific and Chemical Supplies unless otherwise stated.

Cell culture and transfection

NIH3T3, HeLa and COS cells were maintained in DMEM media supplemented with 10% fetal calf serum. P19 cells were maintained in DMEM supplemented with 2.5% FCS and 7.5% new-born calf serum. All media contained penicillin and streptomycin. Undifferentiated P19 cells were induced to differentiate with 0.5 μ M *all-trans* RA for 4 days prior to transfection (19).

Unless otherwise stated, transfections were performed on cell densities of ~30% confluence for undifferentiated P19 cells (seeded at 5×10^5 cells per 5 cm dish), and 60% confluence for RA-treated P19 cells (seeded at 5×10^5 cells) and NIH3T3 cells (seeded at 6×10^5 cells per 5 cm dish). For 5 cm dishes: after overnight growth of seeded cells, the medium was changed 2 h prior to transfection and DNA mixtures were prepared and added to individual cell culture dishes using the BES CaPo⁴⁻ method of transfection; specified plasmid DNAs were balanced for DNA concentration using both UV spectrophotometry and agarose gel analysis and the same batch of BES was used throughout these studies from frozen aliquots. An aliquot of 0.5 μ g of EflacZ was included in

each transfection reaction for β -gal standardization of transfection efficiency. Empty EFplink vector was used to balance DNA concentration in each transfection experiment. Cells were harvested after 48 h for analysis.

Oligonucleotides and plasmid construction

Oligonucleotides used in plasmid construction and electrophoretic mobility shift assay (EMSA) were as follows.

Site-directed mutagenesis. POU domain substitutions > 4POUN-R: GTATGGTTCTGTAGGCGGCG-CCAGAAGG. 4POURR: GATGTGGTTCGAAGATGGTTCTGTAGG. Oct-4 deletions > N-term D61–90 (for Oct-4 7–17 amino acid deletion): +GGCTGGAC-ACCTGGCTGGGGGTGATGGG, –CCCA-ACCCCAGCCAGGTGTCCAGCC. Oct-4 Nco/Bsu join: (+) CATGGCTGGATGTGGACC, (–)TGAGGTCCACATCCAGC, (ii) TCAGGTCCACATCCAGC. (Nco/Bsu join + was duplexed with both Nco/Bsu join – and Nco/Bsu join II – which correspond to duplexes used to produce D4–70 and D4–89 Oct-4 proteins, respectively).

EMSA and cloning target binding sites. H2B: +CTAG-AAGAATCGCTTATGC AAATAAGGTGAAGAT, –CTA-GATCTT-CACCTTATTTGCATAAGCGATTCTT. V κ GG: + TCGATGAGGGTATGCAAG-GTATTAAG, –TCGA-CTTAATACCTTGCATACCCTCA. V κ GT: + TCGATGAGGGTATGCAAGTTATTAAG, –TCGACTTAATAACTTGCATACCCTCA. SEQUENCING >M13 (–40) forward (for pBLCAT-based vectors): GTTTTCCCAGTCACGA. Oct-4POU (for Oct-4 POU domain mutations in any vector): CTAGCATTGAGAACCGTGTG > EFplink forward: TTG-CTTA-CATTTGCTTCTG. EFplink reverse: CCAGGGC-ATTAGCCACAC.

Plasmid construction

Oct-4cDNA was provided by Rosner *et al.* (2) in pCMVOct-4. Oct-1RR was provided by H. Singh, Howard Hughes Medical Institute, University of Chicago. PBLCAT was provided by Luckow and Schutz (20). EFplink vector is described elsewhere (21). pEFLacZ: β galactosidase gene inserted into EFplink for constitutive expression and was kindly provided by S. Walker, Ribotargets Ltd, Cambridge, UK. EVRF0 and pPOU6Vp16 were provided by D. Meijer (22). All plasmid constructs were confirmed via sequencing and protein expression studies were performed where appropriate. CAT reporters > p6WCAT: Multimerized IgH 1W site (6W) inserted 105 bp upstream of minimal TK-promoter TATA box and linked CAT gene. 6W element from pLI4-6xIgH [provided by M. Gstaiger (23)] was ligated into the SalI site of pBLCAT. Constructs were selected where octamer sequence of the 1W elements were orientated 5'–3' on the DNA bottom strand upstream of CAT gene. pV κ GGCAT and pV κ GTCAT GTCAT series: single oligonucleotides corresponding to a variable κ -immunoglobulin promoter element with an AT to GG mutation in the octamer motif (V κ GG) or AT to GT (V κ GT) were ligated into the SalI site in pBLCAT to produce a six-site insertion (p6V κ GGCAT) or seven-site insertion (p7V κ GTCAT), respectively. p6V κ GGCAT was reopened with SalI, and the ligation process was repeated to produce a eleven-site insertion into pBLCAT (p11V κ GGCAT). Oct-4

expression plasmids > pEFOct-4: Oct-4 cDNA from CMVOct-4 was generated by NcoI and EcoRI digestion and ligated into NcoI–EcoRI EFplink to generate an in-frame fusion to the vector Myc epitope sequence. pEFOct-4 Δ N: an AvalI digest of an Oct-4 cDNA was blunt ended by Klenow polymerase fill-in and ligated into SmaI digested EVRF0 to give an N-terminal deletion of sequence corresponding to amino acids 1–105. This Oct-4 sequence was moved into EFplink using a BamHI/XbaI digestion. pEFOct-4 Δ C: an EarI partial digest of Oct-4 cDNA in EfOct-4 was Klenow blunt ended and elucidated with NcoI corresponding to 5' of Oct-4 coding sequence. Fragments were then ligated into NcoI sticky/EcoRI Klenow blunt-ended EFplink to produce clones containing Oct-4 cDNA with deletion of sequence corresponding to amino acids 285–352. pEFOct-4N-R Oct-4 (N51R mutation in the POU homeodomain): an EcoRI Oct-4 cDNA from CMVOct-4 was inserted into pGEM-3Zf(–) from Promega Inc., where the vector PstI site had been removed, XbaI and SpHI, followed by Klenow fill-in of 5' overhangs and 'chewing' of 3' overhang to create blunt ends (using T4 polymerase) respectively, with religation. The resulting plasmid was named pGEMOct-4 and used to make single-stranded bromoxyuridine DNA in the F+ *E. coli* strain CJ236 which was subsequently used for mutagenesis with the POUN-R oligonucleotide. A fragment comprising a 450 bp PstI–NarI fragment encompassing the mutant region was swapped back into pGEMOct-4 to make pGEMOct-4N-R. The Oct-4 carrying the desired mutations was then swapped into EFplink in-frame with the vector myc epitope sequence via an NcoI/EcoRI digest. pEFOct-4RR: as above but using POURR oligonucleotide to mutate pGEMOct-4N-R. pEFOct-4RR Δ N: an AvalI EFOct-4RR fragment was Klenow blunt-end filled and inserted into EFplink (Dam–) cut with ClaI and Klenow blunt-end filled and dephosphorylated. Selected clones contained Oct-4 with deleted sequence corresponding to amino acids 1–105. pEFOct-4RR Δ C: the truncation Oct-4 from pEFOct-4 Δ C (see above) was swapped with the full-length Oct-4 coding sequence into pGEMOct-4 via a NcoI/EcoRI digest making pGEMOct-4 Δ C. Subsequently, a PstI–NarI fragment from EFOct-4RR comprising the mutant part of the binding domain was swapped into the new pGEMOct-4 Δ C to make pGenOct-4RR Δ C. This construct was then used to swap the truncation Oct-4RR sequence into EFplink via an NcoI/EcoRI digest. pEFOct-4RR D7–17: the complementary oligonucleotides Oct-4:7–17 were used. Employing these oligonucleotides, EFOct-4RR was used in the Quickchange mutagenesis kit supplied by Stratagene to produce an Oct-4 mutation with amino acids 7–17 deleted. pEFOct-4RR Δ 2–29: EfOct-4RR was cut at the 5' Oct-4cDNA NcoI and an BamHI internal to Oct-4 cDNA and Klenow blunt-end filled. The resulting digest was religated and clones were selected which contained the Oct-4 sequence which corresponded to deletions of amino acids 2–29. pEFOct-4RR Δ 4–70: the oligonucleotide duplex for Oct-4 Δ 4–70 was used which had 5' NcoI and 3' Bsu36I ends corresponding only to Oct-4 Bsu36I site at codon 70 of Oct-4 sequence. EFOct-4RR was partially digested with Bsu36I, which shows site degeneracy (cutting at Oct-4 positions 259, 310 and 1056) and NcoI corresponding to the Oct-4 5' translation start site. The resulting digest of the correct length was dephosphorylated and used in a ligation with phosphorylated double-stranded

oligonucleotide. pEFOct-4RRΔ4–88: as above but the oligonucleotide duplex Oct-4 Δ4–88 used here corresponding only to 5' NcoI and 3' Oct-4 Bsu36I site at codon 88 of Oct-4 sequence. pEFOct-4RRΔC (7–17, 2–29, 4–70 and 4–88). As above but using pEFOct-4RRΔC as substrate.

Protein expression and binding analysis

Protein extracts. For whole cell protein extracts, frozen cell pellets were placed on ice and resuspended in 2–3 cell pellet volumes of cold whole cell extract buffer (10 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol and protease inhibitors: 10 μg/ml APMSF, 2 μg/ml Aprotinin, 3 μg/ml Antipain, 4 μg/ml Bestatin, 6 μg/ml Chymostatin, 3 μg/ml E-64, 1 μg/ml Leupeptin and 1 mM phenylmethylsulfonyl fluoride). Following 15 min incubation on ice, the samples were microfuged at high speed at 4°C and the supernatant was processed and stored at –70°C. For cell nuclear extracts, pellets were resuspended on ice in 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl and 0.5 mM DTT. After 5 min incubation on ice, NP-40 was carefully added to 0.5% and left for a further 10 min on ice. The homogenate was microfuged for 1 min at mid-speed and the supernatant was removed from the pelleted nuclei. The nuclear pellet was then washed in buffer minus NP-40 and pelleted again. Washed pelleted nuclei were then treated with mammalian whole cell extract buffer as specified above. Prior to EMSA assays or the addition of SDS loading buffer for western blots, supernatants were measured for protein concentration using the BioRad DC system.

Western blots. Typically 60 μg of denatured protein extract was electrophoresed through a 12% SDS polyacrylamide gel onto nitrocellulose membrane. The transferred gel was stained with Coomassie blue to check that transfer had been successful and to confirm protein loading. Blots were processed according to antibody manufacturer's conditions with use of horseradish peroxidase (HRP)-conjugated secondary antibody against primary. Visualization of HRP-driven chemiluminescence was undertaken using X-ray film and a PhosphorImager.

EMSA. Oligonucleotide concentration and integrity was confirmed on an agarose gel prior to use. Complementary oligonucleotides were annealed according to the standard procedures, and double-stranded complexes were precipitated and redissolved in water and DNA concentration measured on a UV spectrophotometer. Oligonucleotide duplexes were also validated on an agarose gel prior to further use. An aliquot of 100 ng of oligonucleotide duplex was radiolabelled using Klenow polymerase and [α -³²P]dCTP to fill-in 5' overhangs according to standard protocols. G50 sephadex resin spin columns were used to extract unincorporated nucleotide. Typically, 12 μg of whole cell or 30 μg of nuclear extract protein and 2 μg poly(dI–dC), poly(dG–dT) or denatured salmon sperm DNA as specified was included in 20 μl reactions with 0.75 ng labelled probe and incubated for 30 min at room temperature in 1× EMSA buffer (50 mM HEPES, pH 7.9, 100 mM NaCl, 20% Glycerol, 400 μg/ml BSA, 0.1% NP-40, 2 mM DTT and 2 mM EDTA). For supershift experiments, antibody was added to reactions after 20 min

and incubated at room temperature for a further 10 min. The reaction mixture was analysed on a 4% non-denaturing polyacrylamide (19:1) gel. Protein–DNA complexes were visualized after autoradiography with or without intensifying screens at –70°C overnight and/or on a PhosphorImager after a 3–16 h exposure.

For competition binding assays and determination of relative apparent dissociation constants (K_d), gel retardations were performed as follows: 0.5 ng of labelled oligonucleotide was added to increasing amounts of cold competitor DNA as specified along with 2 μg poly(dI–dC) in 1× EMSA buffer. At T_0 , a constant protein extract amount was added to reaction tubes on ice, making a final reaction volume of 20 μl, and incubated at room temperature for 30 min.

Transcriptional activation analysis

The following protocols were performed on experimental sets.

Protein extraction. Freeze-thaw lysis was the chosen method of cell disruption for CAT and β-Gal assays. Supernatants were assayed for protein concentration using the DC BioRad protein assay according to the manufacturer's instructions.

The β-galactosidase activity assay. Aliquots containing 100 μg of each cell extract protein sample within an experiment set were aliquoted into separate wells of a microtitre plate and assayed for internal-control β-gal activity. β-gal buffer was mixed with OMPG at a ratio of 4:1, and 250 μl of this mixture was added to individual wells of the microtitre plate containing cell extract aliquots on ice using a 12-tip multipipette to eliminate reaction start-time asynchrony. The microtitre plate was subsequently incubated at 37°C. Using a plate reader set to measure optical density at a wavelength of 420 nm, the microtitre plate reactions were monitored at regular time intervals so that optical density did not fall outside of a time versus optical density (OD) linear relationship. The final value for β-gal activity within an experimental set was calculated by dividing OD₄₂₀ by time (min) and then multiplying by 100. Such β-gal readings were used as an internal control for normalization of transfections or as a guide for the interpretation of results of CAT assays.

CAT assays. A maximum of 70% of whole cell protein extract per transfection plate derived through the freeze thawing process was used in CAT assays for each transfection. β-gal readings for individual transfection reactions were either used to normalize transfections within an experiment as specified in results, or used as a guide for interpretation of results of CAT assays performed for measurement of the transcriptional activity of particular transcription factor systems. Experiments that were not normalized according to β-gal readings were normalized for protein concentration. Acetylation of ¹⁴C chloramphenicol followed by thin-layer chromatography was the method chosen for ascertaining CAT enzyme concentrations and was performed according to the standard protocols. TLC plates were subsequently analyzed using a Fuji FLA2000 PhosphorImager and data analyzed using Aida software.

RESULTS

Altered specificity Oct-4 binds a mutant target site which itself is not efficiently bound by wild-type Oct-4

Oct-4 and Oct-1 share significant sequence homology in their DNA-binding domains and are known to bind very similar motifs within subsets of their respective target genes. It has been previously shown that mutations in the DNA-binding domain of Oct-1 (Oct-1RR) alters its sequence specificity, enabling it to bind efficiently to two artificial recognition sites, ATGCAAGG (GG site) and ATGCAAGT (GT site). Importantly, both sites were shown to bind wild-type protein with a relatively low affinity, albeit with the former site displaying a relative 130-fold lower affinity for wild-type Oct-1 than the latter but the GT site having a relatively higher affinity for Oct-1RR (16). We have transposed these mutations to Oct-4, creating an Oct-4RR (V47R, N51R) mutant and imbedded altered-specificity binding sites into promoter construct to enable exclusion of wild-type Octamer factor contributions in Oct-4 functional studies (Figure 1A and B).

Either GG or GT sites were included in a 25 bp immunoglobulin variable κ -light chain (IgV κ) enhancer element situated on the pBLCAT reporter vector, replacing the single consensus octamer site. (These sequences were designated V κ GG and V κ GT, respectively). This enhancer element contains no other recognized protein binding sites according to the database analysis but has been shown to maintain a highly binding conformation sensitive partnership between the B-cell specific cofactor Bob-1 and the altered-specificity binding Oct-2RR protein (18). Octamer expression constructs used in this study are shown in Figure 1A.

The plasmids EFOct-N51R and EFOct-4RR, along with EFOct-4 (wild-type), were transiently transfected into NIH3T3 cells. Whole cell extracts, normalized for Oct-4 protein concentration via western blot analysis (Figure 1C), were then used in EMSA experiments with respective target binding sites (Figure 1D). As seen in Figure 1D, binding of Oct-4RR and Oct-4N51R to the wild-type octamer motif ATGCAAAT (Histone 2B promoter-based probe) was similar to that seen for wild-type Oct-4. Conversely, however, wild-type Oct-4 bound only very weakly to the V κ GG site, compared with Oct-4RR and Oct-4N51R and in comparison with binding to the wild-type site (Figure 1D, lanes 2–4 compared with lanes 7–9). The relative reduction in binding activities between wild-type and V κ GG sites, which was \sim 200-fold, 20-fold and 4-fold for wild-type Oct-4, Oct-4N51R and Oct-4RR, respectively, suggests that binding of Oct-4RR to V κ GG sites is significantly higher than for Oct-4N51R binding to the same site.

As can be seen in Figure 1D (right hand panel), Oct-4RR binds more strongly to V κ GT than to V κ GG sites (lane 7' compared with lane 12'). However, unlike for the V κ GG site, wild-type Oct-4 also binds significantly to the V κ GT site (Figure 1D, lane 2' compared with lanes 6' and 10'). Wild-type Oct-1, similarly to Oct-4, also bound more strongly to V κ GT than to V κ GG sites, although this was only obvious after over-exposure of the EMSA gel. Oct-1RR was included as a control and mirrored Oct-4RR binding data in terms of the relative affinities for wild-type and altered-specificity sites, in comparison with wild-type protein (Figure 1D, lanes 4', 8' and 13').

In order to obtain more quantitative binding data describing the difference in affinities for wild-type and mutant proteins

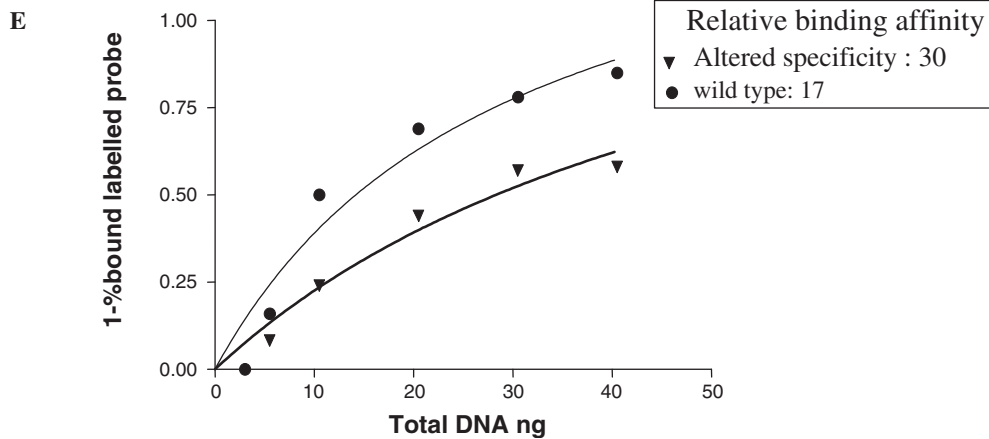
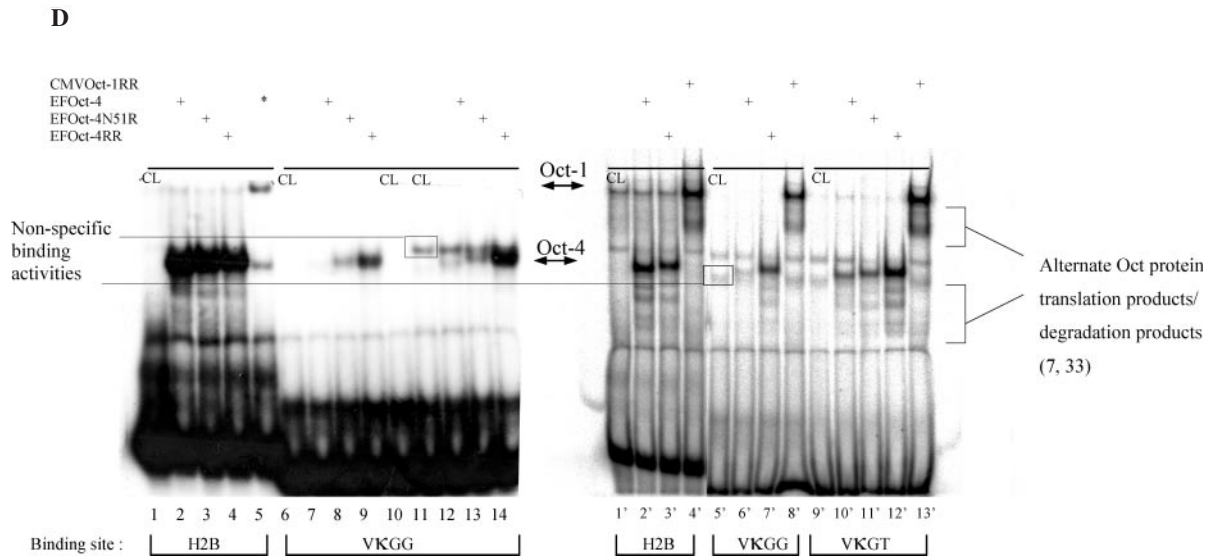
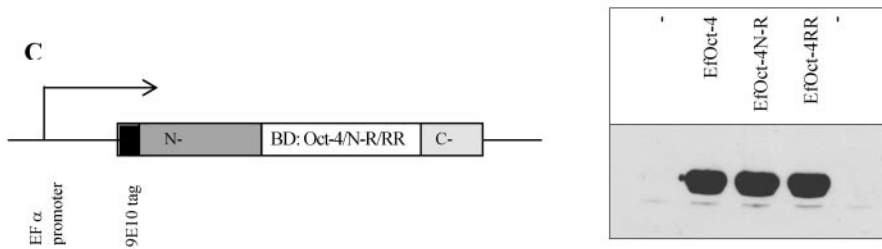
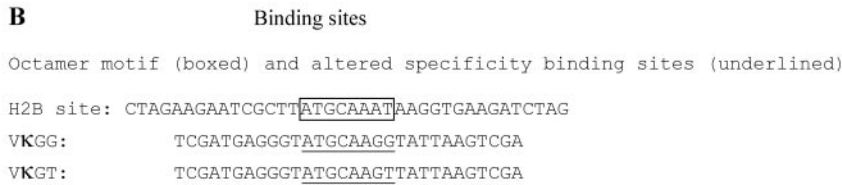
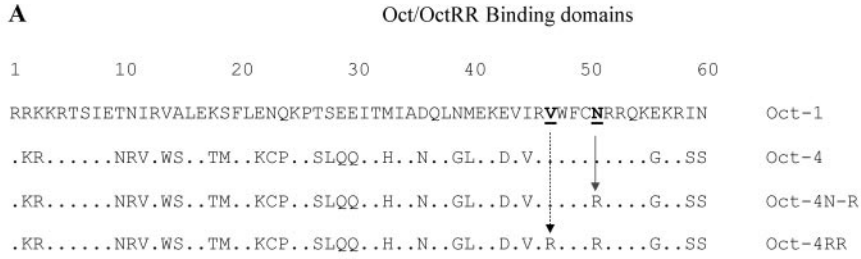
for their respective recognition sites, oligonucleotide competition binding assays were performed. Figure 1E shows the binding curves obtained using whole cell extracts in which Oct-4 and Oct-4RR had been over-expressed. The relative binding affinity of Oct-4RR for the V κ GG site was shown to be \sim 2-fold lower than the affinity of Oct-4 for the wild-type octamer site. This, along with evidence of poor binding of endogenous protein to the V κ GG site, suggested to us that we could confidently undertake functional studies of Oct-4 using the altered-specificity system. The GG site was primarily chosen for further study with Oct-4RR, although the GT site was also used as the functional relevance of higher wild-type protein binding was unknown and as it resembled the sequence ATGCTAGT, a natural target for Oct-4 found in the *UTF-1* gene enhancer element (24).

Transiently expressed Oct-4RR, but not endogenous or exogenous Oct-4, promotes gene expression through the altered-specificity binding sites

The functionality of monomeric Oct-4RR was demonstrated as a 'stand-alone' transcription factor in undifferentiated ('pluripotent') P19 cells using V κ GG (in p6V κ GGCAT) and V κ GT (in p7V κ GTCAT) site-containing reporter constructs (Figure 2A and B). Relative transactivation of CAT reporter gene above basal activity was measured at 10-fold for Oct-4RR/V κ GG and 5-fold for Oct-4RR/V κ GT protein/reporter vector combinations, which was consistent with the binding data presented here (Figure 1D). No transactivation was demonstrated in the absence of target sites (pBLCAT vector). Importantly, over-expression of wild-type Oct-4 was not associated with transactivation through either binding site. However, given the higher affinity binding of the Oct-4 mutant proteins and wild-type Oct-4 for V κ GT over V κ GG probes, these results were unexpected to some degree and may indicate greater interference from endogenous POU proteins *in vivo* or a functionally sub-optimal protein–DNA conformation specific to the V κ GT site. Transcriptional activation associated with the 6W element (in p6WCAT), which contains the wild-type octamer motif, was over 40-fold that of empty reporter vector (pBLCAT) and significantly higher than described above for Oct-4 mutants through V κ GG and V κ GT sites. This suggests that promoter context or structural conformation may be important in presenting optimal Oct-4 transactivation function. Consistent with binding data, Oct-4N51R displayed at least half the transcriptional activity as Oct-4RR in all cases and was therefore not studied in any greater detail. The non-optimal V κ GT site was also withdrawn from further detailed studies.

Oct-4RR activates transcription to an equivalent amount in undifferentiated and differentiated P19 cells through V κ GG sites

To assess any relative differences in Oct-4RR transactivation potential between undifferentiated (P19U) and differentiated P19 (dP19) cells, a range of expression vector concentrations were used in transient transfection experiments. Oct-4RR activates reporter gene expression in both P19U and dP19 cells to a comparable degree (Figure 3D). For both cell states, the maximum transcriptional activity associated with Oct-4RR was at low vector concentrations (100–250 ng range). Total



amounts of transfected DNA were equivalent for all transfection experiments. Western blot and EMSA analysis showed an approximate 3- to 4-fold higher concentration of Oct-4RRR per μg of protein in dP19 compared with P19U cell extracts (Figure 3A and B). This reflects the relative transfection efficiencies of P19U and dP19 cells (25 and 42%, respectively) and is confirmed by control β -galactosidase (β -Gal) data (Figure 3D). (Transactivation was measured against internal controls within each experiment, allowing accurate comparison of transactivation levels between each cell type).

Importantly, transient Oct-4RRR concentrations studied in these experiments matched endogenous Oct-4 protein levels within the optimal range of vector concentrations used in functional studies, and thus can be considered as physiologically relevant (see Figure 3C).

EMSA data confirmed the presence of endogenous Oct-4 in P19U cells and that its expression was extinguished following cell differentiation (Figure 3B). In accord with previous studies (9), the transactivity associated with the 6W enhancer, which contains the consensus octamer motif, was relatively high in P19U cells, compared with differentiated cells which have been transfected with Oct-4 expression vector (Figure 3D and E). The 6W enhancer therefore demonstrates a clear cell-specific activity which is not seen for V κ GG sites in response to Oct-4RRR expression.

Oct-4RRR N-terminus remains the predominant activation domain through differentiation

The next experiment set out to determine which regions of Oct-4 are the most important for transactivation function in pluripotent cells and whether such 'activation domains' function similarly in differentiated cells. Using existing information on Oct-4 protein structure, extensive Oct-4RRR C- and N-terminus activation domain deletions were constructed and used in cotransfection experiments with appropriate reporter vectors (Figure 4). Primary analysis of CAT data revealed no cell-specific activation domain usage (Figure 5). Further data analysis indicated that in an Oct-4-native, pluripotent environment and in a differentiated cellular environment, the N-terminus is the more powerful of the Oct-4 activation domains in a stand-alone situation, while the C-terminus displayed a lower but still significant transactivity (Figure 5A). Protein expression and competition binding assays indicated that protein concentration and binding affinity of each deletion mutant were approximately equal and therefore not a contributing factor in the differing transactivation

levels seen here (Figure 5B). Importantly, the same deletions of wild-type Oct-4, when assayed using a wild-type site-containing reporter construct, display equivalent transactivation profiles to those of Oct-4RRR deletion experiments (Figure 5C, i). This result suggests that no fundamental structural or functional change is occurring in the Oct-4 mutant proteins, as a consequence of modification of their DNA-binding domains.

To determine whether there was any cell-specific usage of subregions of the more powerful N-terminus activation domain, a series of truncation proteins were constructed where deletions of increasing size from the 5' end of the N-terminus were introduced (Figure 4). Such truncation proteins were also studied with and without intact Oct-4 C-terminus in order to determine whether there was any dependence on the C-terminus activation domain for Oct-4 function. Inference from the results of N-terminus deletions of amino acids 1–28, 7–17 and 3–70, shown in Figure 6, suggests that the N-terminus subdomain containing amino acids 28–70 is essential and sufficient for full N-terminus Oct-4 activity, while again exhibiting no cell-specific preference. Deletion of the C-terminus caused a drop in transactivity across the board, which was consistent with previous data showing that this domain possessed a significant non-cell-specific transactivation function. No dependence on the C-terminus was exhibited by any of the active N-terminus domains.

DISCUSSION

Using an altered-specificity binding strategy, it is demonstrated here that Oct-4RRR as a 'stand-alone' transcription factor displays the same transactivation profile in both pluripotent and differentiated cells, thus casting doubt over a previously hypothesized functional partnership between Oct-4 and an unidentified pluripotent cell-specific transcription cofactor in such a promoter context. The possibility that any potential cofactor interaction might be highly sensitive to binding context or subtle changes in binding conformation, and is thus not observed when using non-wild-type Oct-4, must be acknowledged. However, given the overall homology of the 4RR POU domain to that of wild-type protein and evidence for equivalent functionality to wild-type protein shown here, this explanation is unlikely.

Despite the longstanding postulated existence of an Oct-4 cofactor specific to pluripotent cells (8,9,13), there is as yet no evidence presented which identifies such a factor, although

Figure 1. Binding of wild-type Oct-4, Oct-4N51R and Oct-4RRR to altered-specificity binding sites V κ GG and V κ GT. (A and B) Mutant binding domains and target sites are detailed. (C) COS cells were transfected with 3 μg of indicated plasmids. Western blots were performed in duplicate using mouse antibody against the myc epitope, which was fused to the N-terminus of all the proteins. (D) Using protein expression data from western blots, Oct-4-type expression was normalized and a maximum of 12 μg of protein extracts were used in each EMSA reaction for lanes 1–10 and 1'–13' and a maximum of 24 μg of protein for lanes 10–14. In lane 5 (asterisk), 12 μg of HeLa cell extract for an EFOct-4 transfection replaces COS cell extract. Each reaction also included 2 μg of salmon sperm non-specific DNA and 1 ng of radio-labelled binding site as specified. 12 μg of cell protein extract from a transfection of CMVOct-1RR plasmid was also used in these EMSA experiment. EMSA experiments are representative of duplicated experiments. Oct-1/1RR and Oct-4/4RRR/4N-R probe complexes are indicated. CL refers to extracts from transfections of empty vector (EFpLink) control. Bands that are boxed were demonstrated by subsequent competition studies to be non-specific. (E) Relative differences in binding affinity between altered-specificity and wild-type binding systems were derived from triplicate competition EMSA experiments and are illustrated using a representative set of data. As specified in Materials and Methods, fixed concentrations of Oct-4 and Oct-4RRR cell protein extract, normalized for Oct-4-type protein concentration according to western blot data, were used with 1 ng of H2B or V κ GG labelled probe respectively and a range of non-labelled self competitor oligonucleotide (2.5–40 ng). All competition EMSA reactions contained 2 μg poly(dI-dC) competitor DNA. Band intensity relating to Oct-4:H2B/Oct-4RRR:V κ GG complexes was measured as a proportion of 1 (*P*-values; a value of 1 being attributed to band intensity for binding reactions with 2.5 ng self competition). 1 minus *P*-values were plotted against total oligonucleotide using Graph Pad (Prism Ltd) software package. KM refers to relative binding equilibria.

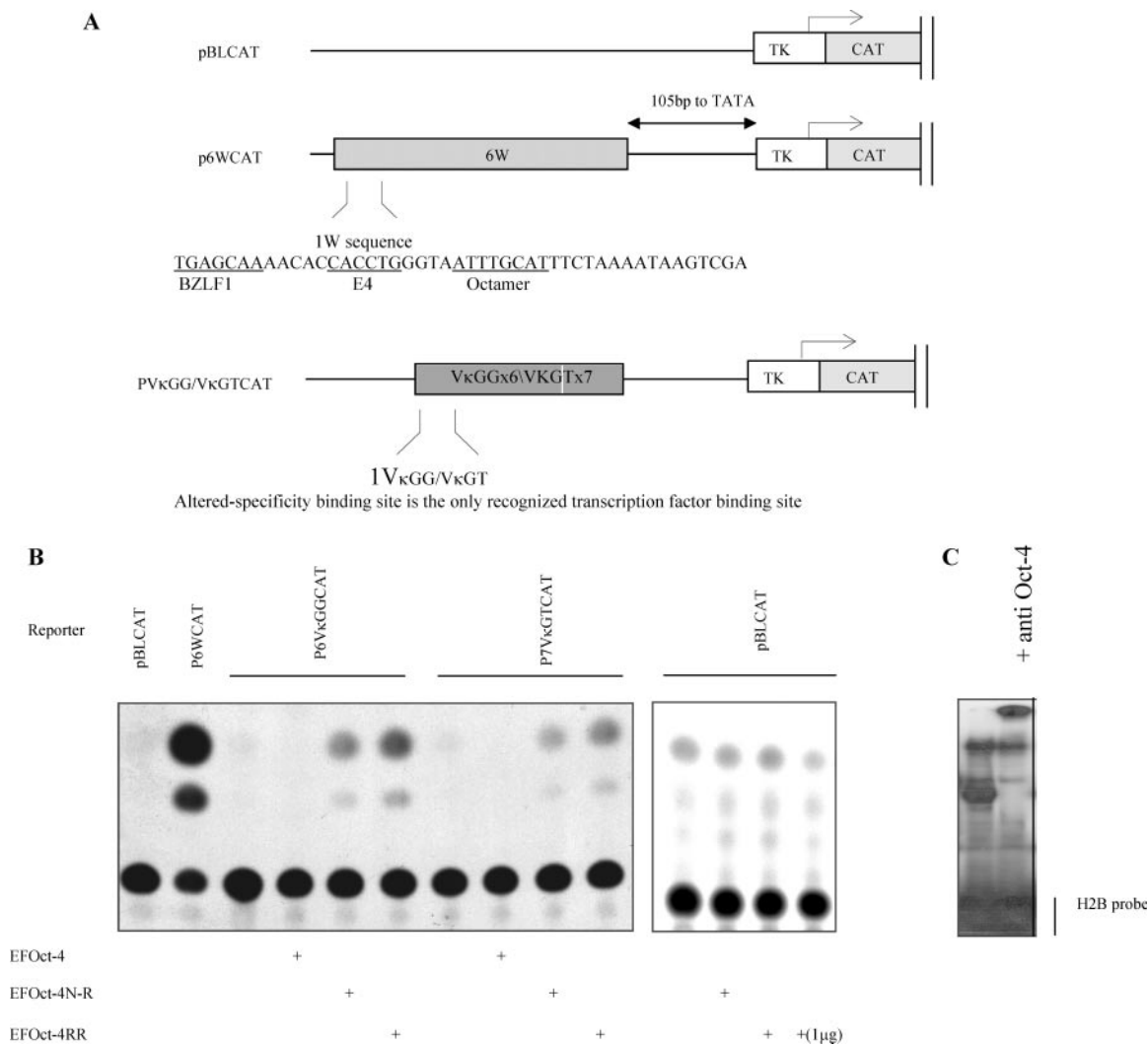


Figure 2. VκGT altered-specificity binding site displays a weaker enhancer function than VκGG. (A) Target constructs are detailed. The 6W sequence is derived from Gerster *et al.* (34). (B) and (C) Undifferentiated P19 cells were transfected with specified plasmids. A total of 3.7 μg DNA was used in each reaction using empty EFlink plasmid for normalization. 100 ng of reporter plasmid was used in each transfection and 100 ng of expression vector (EFOct-4/Oct-4N-R/Oct-4RR) unless otherwise specified. CAT assays were subsequently performed on sample extract normalized for β-gal activity within an experiment. Data presented here are representative of experiments performed in triplicate. (C) For a representative experiment, EMSA was performed on whole cell extract made from 20% of cells from transfection of pBLCAT only. Polyclonal rabbit anti-mouse Oct-4-specific antibody was used in EMSA supershift experiments.

Oct-4 has been shown to interact with a number of proteins ubiquitously. Phage display and GST-pull down data indicate that Oct-4 interacts directly with the ubiquitous high mobility group protein HMG-1, which cooperates with Oct-4 to some degree to activate transcription in P19U cells (25). The same study indicated that HMG-1 and two other minor Oct-4 interacting proteins were unlikely to account for an apparent undifferentiated P19 (P19U) cell-specific Oct-4 transactivity. Oct-4 has also been shown to directly interact with a single pluripotent cell-specific protein, FoxD, in GST-pull down assays (26), although in this case Oct-4 functions as a weak corepressor, affecting FoxD transactivity in *FoxA1/FoxA2* promoters. In this case, FoxD was not implicated as a factor which regulates Oct-4 activity on Oct-4 target promoters and as such is not a realistic candidate for a cell-specific Oct-4 cofactor. Moreover, an alternative interpretation for such Oct-4-mediated repression is that over-expression of

Oct-4 to high levels leads to general transcription repression, perhaps through sequestration of general transcription factors or other non-specific phenomena. This effect is also noted for Oct-4 both in this current work and in data from other studies (15).

Bearing in mind the importance of an intact protein for proper Oct-4 function, studies here focused on minimal modifications to the Oct-4 binding domain that altered its sequence specificity to allow recognition of a custom binding site, which itself was not effectively bound by wild-type POU proteins, while maintaining a wild-type binding conformation. Thus, a relevant and essentially 'stand-alone' assessment of Oct-4 transactivation function could be made.

An important first step in this work was to demonstrate that the altered-specificity system originally used in the study of Oct-1 function would translate to an Oct-4 based system. Binding analysis clearly showed that endogenous Oct-4 did

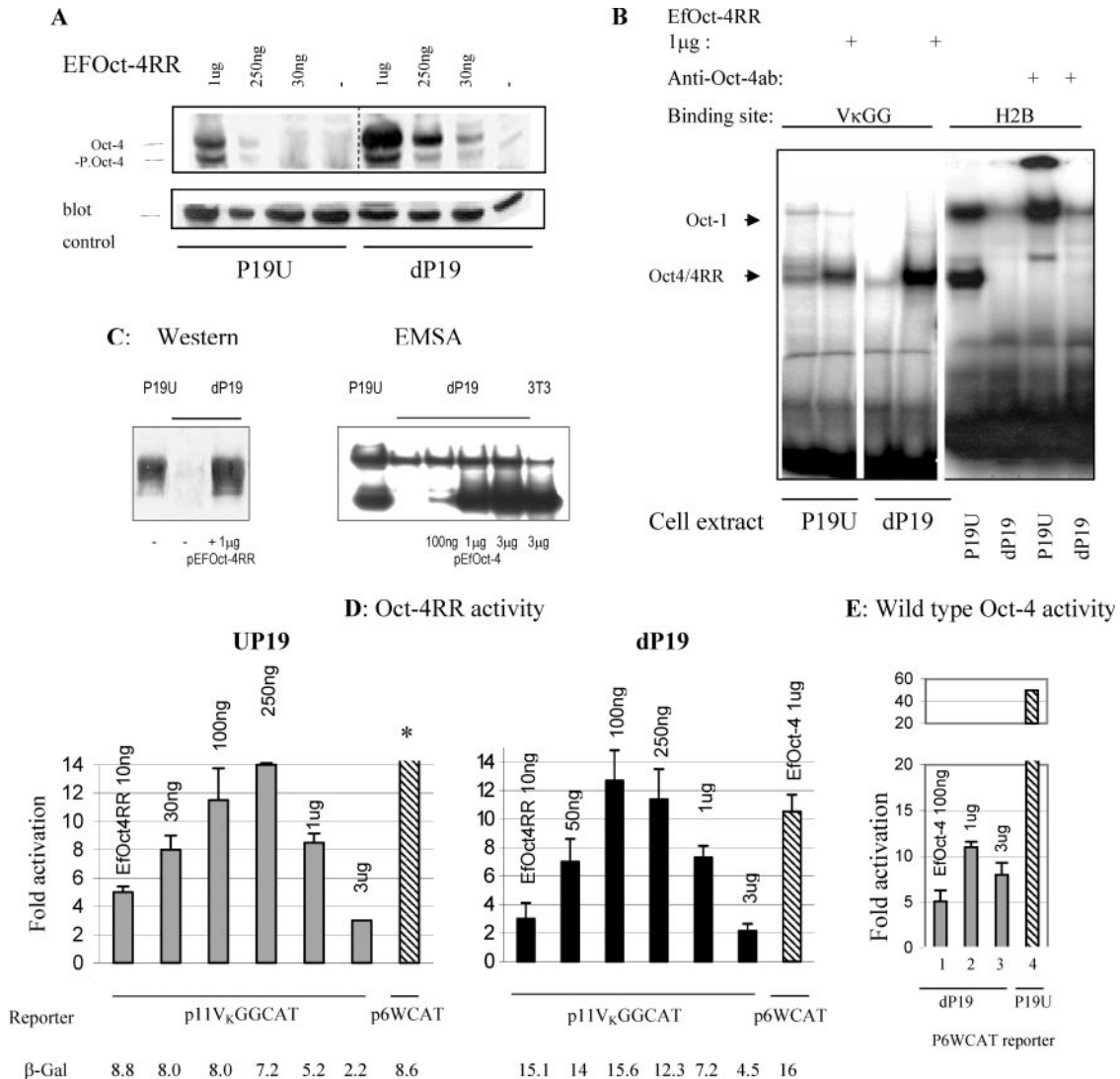


Figure 3. Oct-4RR has equivalent transactivation potential in undifferentiated and differentiated cellular environments. Undifferentiated P19 (P19U) cells or P19 cells induced to differentiate with 0.5 μ M RA for 4 days (dP19 cells) were transfected with specified plasmids. (D and E) Reporter plasmid was transfected at 120 and 60 ng for P19U and dP19, respectively. Extract protein concentration was measured and half as much total protein was used in experiments for dP19 than P19U. Fold activation refers to the proportional increase in reporter gene expression in response to Oct-4RR, relative to the basal transcriptional activity of reporter vector alone. Averages and standard errors are shown for experiments repeated three times. Internal control β -gal readings, like CAT activity, seen at the higher end of Oct-4RR expression vector concentrations were seen to be depressed, probably due to squelching of transcription associated molecules or as a consequence of large amounts of mRNA overwhelming the translation process. As such, β -gal data were not used to normalize CAT experiments, but rather plotted as a separate set of representative readings for reference. Asterisk indicates over 40-fold activation of reporter gene. (E) For experiments using wild-type Oct-4, β -gal readings are used to standardize transcription levels. (A and B) For two of the three experiment repetitions, 20% of transfected cells were used to make whole cell extracts for EMSA and western blot experiments in (A and B). (A) An aliquot of 100 μ g of protein extract was included in western blot using the anti-myc antibody against the epitope translated at the N-terminus of Oct-4RR. The predicted Oct-4 band of 38–40 kDa is shown. A blot control protein for loading and transfer normalization is shown and represents endogenous protein cross-reacting with antibody used. (B) Each EMSA reaction included 20 μ g of protein extract, specified probe binding site and 1 μ g of poly(dI–dC) as non-specific competitor. (C) Following a separate set of transfection experiments using specified plasmids, western blots were performed using antiOct-4 antibody and EMSA experiments undertaken to examine wild-type Oct-4 binding to consensus octamer site in the H2B element. At 1 μ g of expression vector, bound and free Oct-4 protein extract levels in dP19 cells were approximately twice that of endogenous Oct-4 levels in P19U cells. After transfection efficiency is taken into account, cellular Oct-4RR concentrations at 250–500 ng levels of expression vector were predicted to be approximately equivalent to endogenous Oct-4 levels in both cell types.

not bind significantly to the VκGG site, whereas the Oct-4RR protein bound relatively strongly to this sequence, indeed more strongly than reported for Oct-1RR mutants in similar studies (16) and with only a slight drop in binding affinity compared with wild-type Oct-4 for the consensus octamer motif. Such differences between related factors, however, may be a consequence of general differences in binding affinity between

Oct-4 and Oct-1 (27), or as a consequence of using cell extracts rather than recombinant protein. For example, Oct-4 is shown to bind as a dimer on the composite osteopontin PORE enhancer using cellular extracts, but not using recombinant protein (28).

In line with binding data, endogenous wild-type Oct-4 or transiently expressed Oct-4 was unable to activate episomal

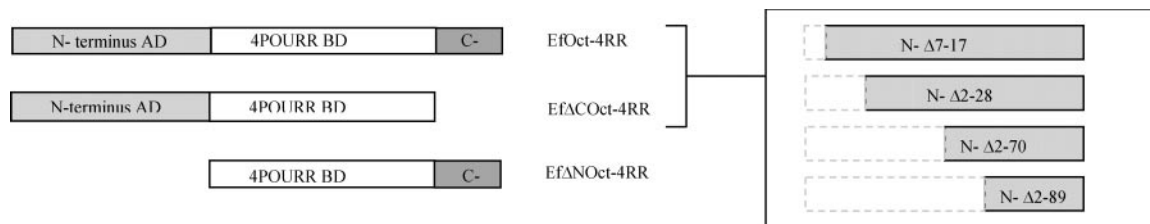


Figure 4. An extended series of Oct-4RR N-terminus deletions were made on Oct-4RR with or without C-terminus.

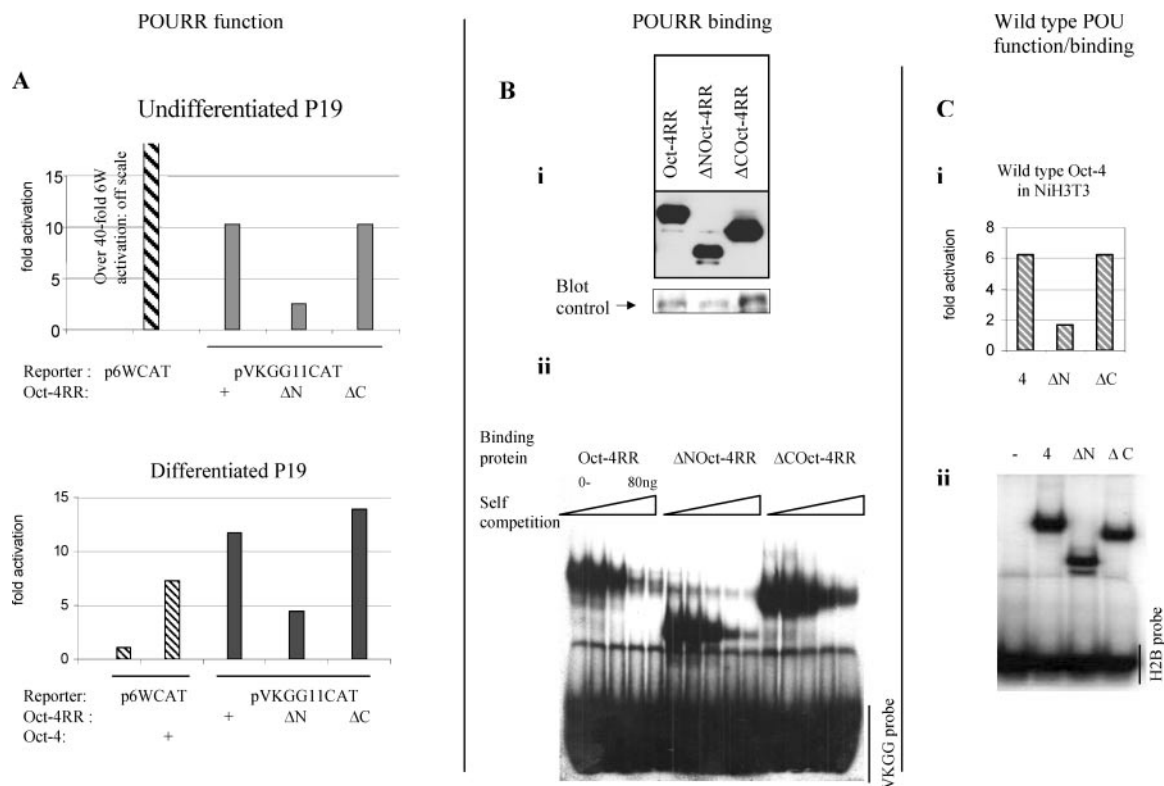


Figure 5. Oct-4RR N-terminal and C-terminal domain truncations exhibit a similar pattern of transcriptional activity in P19, dP19 and NIH3T3 cells. (A) Undifferentiated P19 cells or P19 cells induced to differentiate with 0.5 μ M RA for 3 days and NIH3T3 cells were transfected with specified plasmids. Reporter vector was included at 120, 60 and 250 ng for P19U, dP19 and NIH3T3, respectively, and 250 ng and 1 μ g of Oct-4RR and Oct-4-based plasmids was used in transfections of P19 and NIH3T3 cells, respectively. Cells were harvested after 48 h post transfection and cell extracts for CAT assays and EMSA experiments were made from 70 and 30% of cells, respectively. Data are representative of experiments repeated three times and the mean values of at least two independent transfection experiments are plotted. Fold activation refers to a multiple of background CAT activity seen for reporter vector. Internal control β -gal readings were used to standardize transfection efficiency and protein expression/binding is assessed in (B and C). Western blots were performed against the translated myc epitope in B using 100 μ g of nuclear protein extract and used to balance protein input in EMSA experiments in (C, i). For wild-type protein binding in (C, ii), 20 μ g of protein extracts are used directly in EMSA experiments.

reporter gene expression from upstream $\text{V}\kappa\text{GG}$ binding sites, whereas transient expression of Oct-4RR lead to significant upregulation of this reporter gene construct. Maximum activation of reporter gene expression by Oct-4RR from upstream target sites was not significantly different between undifferentiated embryonic carcinoma cells (P19 cells), where Oct-4 is normally expressed, and differentiated P19 cells where Oct-4 expression is no longer seen. Moreover, as there was no indication of any significant trend of enhanced Oct-4RR transcriptional activity specific to undifferentiated cells over a wide range of cellular protein concentrations, and which cover physiological Oct-4 concentrations, then it is unlikely

that Oct-4RR has any cell-specific activity through the $\text{V}\kappa\text{GG}$ reporter construct. This result is in clear contrast to published evidence and our own observations, which show wild-type Oct-4 is responsible for a significantly higher level of transcriptional activation in undifferentiated EC cells compared with differentiated cells, through consensus octamer binding sites (1,7).

The altered-specificity binding site is based on the $\text{I}\kappa\text{K}$ light chain promoter, which does not possess any consensus transcription factor binding sites other than the mutant octamer motif. The 6W element used predominantly in previous studies of Oct-4 cell-specific activity, however, contains a wild-type

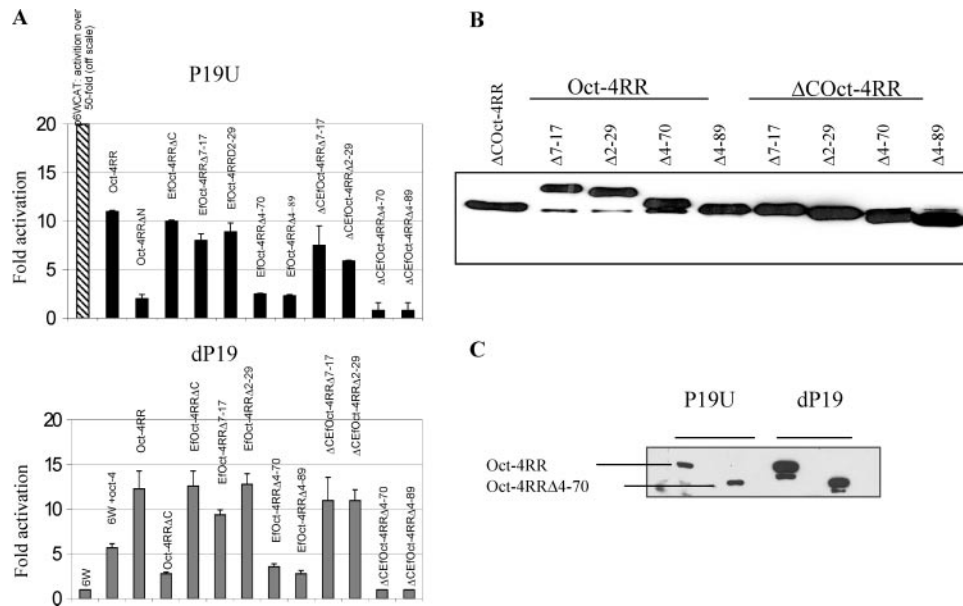


Figure 6. Extended deletions of Oct-4RR N-terminal show no cell specificity for transcriptional activity. (A) Undifferentiated P19 cells or P19 cells induced to differentiate with 0.5 μ M RA for 3 days were cotransfected with 250 ng of specified Oct-4RR plasmids and pV κ GG11CAT reporter plasmid (reporter plasmid was transfected at 120 ng for P19U and 60 ng for dP19 throughout). pWCAT reporter plasmid was also used in control transfection experiments were specified. Cells were harvested after 48 h post transfection and whole cell extracts made for CAT assays. CAT assays were subsequently performed on sample extract normalized for β -gal activity within an experiment for each cell type. Figure is representative of data repeated at least three times with averages and standard errors shown. (B and C) In parallel transfection experiments using 1 μ g of specified expression vectors, nuclear protein extracts were made and western blots were performed against the translated myc epitope. Blots are representative of duplicated data.

octamer motif which is a target for Oct-1 and Oct-6 as well as Oct-4, and has adjacent binding sites for β H β LH transcription factors (7–9,15). As such, the 6W enhancer allows multiple factor interactions that could potentially modulate Oct-4 binding affinity or activity. Interestingly, a 6W element lacking a functional β H β LH binding site and a H2B promoter element, which contains a consensus octamer motif only, both show a significantly lower activity in undifferentiated P19 cells than the intact 6W element and are not activated at all by exogenous Oct-4 in differentiated P19 cells (data not shown). This suggests a fundamental involvement of the β H β LH site in regulating Oct-4 function, but still points towards a cell-specific regulation of Oct-4 to some degree. However, no evidence of proteins binding β H β LH sites was seen here or elsewhere [(29) and data not shown]. As such, the relationship between Oct-4 and the β H β LH binding site in the 6W element remains unknown.

One explanation as to why no cell-specific Oct-4RR activity is seen in these studies compared to wild-type Oct-4, is that other POU proteins may ordinarily contribute to/interfere with wild-type Oct-4 function when bound to octamer motif themselves. For example, it has been shown that Oct-4 can be recruited to promoters by Oct-1, with a positive effect on transcription activation (29). In this respect, it is interesting to note that our binding data suggests higher levels of Oct-1 binding to H2B octamer motif for undifferentiated P19 cells than differentiated cells (Figure 3B). An alternative model for the regulation of Oct-4 function is a direct competition between Oct-1 and Oct-4 for octamer site binding. Oct-4 transcriptional activation studies by several laboratories indicate that cellular Oct-1 does not activate transcription through octamer motif DNA to a significant degree when compared

with Oct-4 in differentiated cells (1,2,7,17,30). Thus, if Oct-1 has a different distribution, binding profile or transcription regulation capability between different cell states, this would translate to an apparent cell-specific Oct-1 repression/enhancement of Oct-4 transactivation in differentiated/undifferentiated cells, respectively. A recent investigation into Oct-1 nuclear localization in *Xenopus* embryos showed that a higher level of Oct-1 protein localized to the nucleus following cell differentiation, possibly supporting the repression model of Oct-4 regulation (31). However, no such evidence for an Oct-1 cell-specific distribution pattern was seen here using whole cell or nuclear protein extracts, although distribution differences that are particular to a specific cell-cycle stage or due to nuclear compartmentalization are unlikely to be picked up in such assays.

Deletion studies allowed P19U cell-specific activation domain usage to be detected that might have been masked in studies using full-length protein, due for example to a general non-specific Oct-4RR transactivity that is particular to differentiated cells. Consistent with studies of intact Oct-4RR protein, no cell-specific activation domain usage was noted here. Accordingly, any cell specificity attributed to Oct-4 must be a function of the wild-type POU domain binding conformation or binding site context.

The N-terminus was shown to be the predominant Oct-4RR activation domain with the C-terminus exhibiting a lower but still significant transactivity. This result was consistent with the findings of Niwa *et al.* (13), who indicate that the Oct-4 C-terminus can complement deletion of the N-terminus, and vice versa, to maintain the Oct-4-dependent pluripotent phenotype. The transactivation function of the N-terminus required amino acids 30 to 70. Such a finding has previously been seen

to a limited degree using Oct-4 N-terminus:c-Jun fusion proteins in P19U cells and a reporter based on the c-Jun binding site (1,32). As such, this shows that the Oct-4RR POU and c-Jun binding domains maintain the same functional relationship between the Oct-4 N-terminus and the general transcription machinery to some degree. However, data for the Oct-4 N-terminus:C-Jun fusion protein studies indicates that amino acids 1–36 are needed for complete N-terminus function whereas we show that deletion of amino acids 2–29 from Oct-4RR has little or no effect on protein transactivation function. As no obvious functional motifs are apparent between amino acids 29 and 36, then the conflicting results seen between c-Jun and Oct-4RR N-terminal deletion studies may point to an inter-regulatory function between the Oct-4 N-terminus activation domain and Oct-4 binding domain, which is abrogated when the c-Jun binding domain is used to target the N-terminus to a promoter. As introduced earlier, such a specific dependence for the Oct-4 POU domain has been previously noted for the Oct-4 C-terminus activation domain which was shown to display a HeLa cell-specific activity from 6W enhancer DNA only when linked to its cognate binding domain (15).

In conclusion, the results presented here demonstrate that an altered-specificity binding Oct-4 protein can be successfully used in transactivation studies in an Oct-4-native environment, without interference from wild-type octamer factors, while maintaining the characteristics of promoter-bound wild-type protein. Using this system, we have been able to show that Oct-4 has an equivalent transactivation function in pluripotent and differentiated cellular environments when presented as a stand-alone transcription factor. These results therefore cast doubt on a previously postulated pluripotent cell-specific Oct-4 function on equivalent wild-type promoters that is gained through the recruitment of an unknown E1A-like transcription cofactor specific to these cells. We now propose that an observed Oct-4 cell-specific activity may be the product of additional binding of transcription factors, such as Oct-1, that either interfere with or modulate Oct-4 activity and which themselves have either a different distribution or activity between cell types. These results should provide further insight into the functional attributes of Oct-4, and its role in stem cell biology.

ACKNOWLEDGEMENTS

Funding to pay the Open Access publication charges for this article was provided by King's College London.

Conflict of interest statement. None declared.

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