The NRF-1/ α -PAL transcription factor regulates human *E2F6* promoter activity

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E2F6 is widely expressed in human tissues and cell lines. Recent studies have demonstrated its involvement in developmental patterning and in the regulation of various genes implicated in chromatin remodelling. Despite a growing number of studies, nothing is really known concerning the *E2F6* expression regulation. To understand how cells control *E2F6* expression, we analysed the activity of the previously cloned promoter region of the human *E2F6* gene. DNase I footprinting, gel electrophoretic-mobility shift, transient transfection and site-directed mutagenesis experiments allowed the identification of two functional NRF-1/ α -PAL (nuclear respiratory factor-1/ α -palindrome-binding protein)-binding sites within the human *E2F6* core promoter reg-

ion, which are conserved in the mouse and rat *E2F6* promoter region. Moreover, ChIP (chromatin immunoprecipitation) analysis demonstrated that overexpressed NRF-1/ α -PAL is associated *in vivo* with the *E2F6* promoter. Furthermore, overexpression of full-length NRF-1/ α -PAL enhanced *E2F6* promoter activity, whereas expression of its dominant-negative form reduced the promoter activity. Our results indicate that NRF-1/ α -PAL is implicated in the regulation of basal *E2F6* gene expression.

Key words: chromatin immunoprecipitation (ChIP), E2F gene, gene regulation, NRF-1/ α -PAL (nuclear respiratory factor-1/ α -palindrome-binding protein), promoter analysis.

INTRODUCTION

E2F6 is a member of the E2F transcription factor family that plays a key role in the regulation of cellular proliferation and differentiation via target genes involved in DNA replication, DNA repair, cell cycle control and apoptosis [1–3]. In mammals, the E2F family consists of seven E2F members (E2F1–E2F7) and two distantly related DP members (DP1–DP2), which form heterodimers to generate functional E2F complexes and regulate transcription from a consensus sequence TTTSSCGC [4,5]. E2Fs members can be grouped into four groups based on their structure, affinity for pRB (retinoblastoma susceptibility protein) family members (pRB, p107 and p130) and functions. E2F6, which lacks the pocket protein-binding domain and the acidic transactivation domain common to E2F1–E2F5 proteins, forms the third E2F subgroup [6–8]. While E2F1–5 proteins can mediate either activation or repression depending upon which proteins associate with their C-terminal domain, E2F6 and E2F7 are only known to mediate repression of E2F target genes. The transcriptionally repressive properties of E2F6 are mainly supported by its C-terminal repression domain [8], which binds components of the mammalian PcG (polycomb group proteins) complex and recruit histone deacetylase activity [9,10]. Overexpression of *E2F6* suppresses the E2F activity on *E2F* reporter constructs and is able to repress the activity of synthetic reporter constructs when fused to the corresponding heterologous DNA-binding domain [7,8]. Moreover, ectopic expression of *E2F6* leads to the accumulation of cells in S-phase, and delays re-entry of quiescent cells into the cell cycle [8,11]. In addition to potential roles in cell proliferation and quiescence depending of the cell type, E2F6 protein is critical for developmental patterning, as revealed by knock-out experiments. Mice lacking *E2F6*, similarly to some PcG mutant mice, display posterior homoeotic transformations of the

axial skeleton [12]. While these transformations arise generally when *Hox* genes are mis-expressed as a result of a lack of PcG-dependent repression, it was tempting to speculate that E2F6 contributes to *Hox* genes promoter regulation. Nevertheless, recent characterization of E2F6 target genes using a combination of ChIP (chromatin immunoprecipitation) and genomic microarrays failed to identify *Hox* genes, but identified numerous genes involved in tumour suppression and maintenance of chromatin structure (*brca1*, *ctip*, *art27*, *hpl-alpha*, *rbp48*) [13]. Although considerable information is now available concerning the mechanism underlying *E2F6* repression, little is known about the regulatory mechanisms that might affect *E2F6* expression. We have therefore recently cloned and characterized the promoter region of the gene encoding human and mouse *E2F6* [14,15]. To clarify the molecular mechanisms controlling *E2F6* expression, we analysed more precisely the human *E2F6* promoter activity and identified the NRF-1/ α -PAL transcription factor as an *E2F6* promoter regulator.

NRF-1/ α -PAL (nuclear respiratory factor-1/ α -palindrome-binding protein), named NRF-1 hereafter, was concomitantly characterized as an activator of the eukaryotic initiation factor 2 α [16] and cytochrome *c* expression [17]. It was subsequently found to act on many nuclear genes required for mitochondrial respiratory function (reviewed in [18]). This predominant role was confirmed by disrupting the *NRF-1* gene in mice, which results in a peri-implantation lethal phenotype and a striking decrease in the mitochondrial DNA content in *NRF-1*^{−/−} blastocysts [19]. More recently, the regulation by NRF-1 of the chemokine receptor CXCR4 [20,21], the GluR2 subunit of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subtypes of glutamate receptors [22], the CD155 human polio virus receptor [23] and the CD47 or IAP (integrin-associated protein) [24] has been demonstrated, suggesting a broad role for NRF-1 in the

Abbreviations used: ChIP, chromatin immunoprecipitation; DBD-Gal4, DNA-binding domain of Gal4; eIF, eukaryotic translation-initiation factor; EMSA, electrophoretic mobility-shift assay; FMR1, fragile X mental retardation 1; ICAM-1, intercellular cell-adhesion molecule 1; IvT, *in vitro* translated; NRF-1/ α -PAL, nuclear respiratory factor-1/ α -palindrome-binding protein; DN-NRF-1, dominant-negative NRF-1; PcG, polycomb group proteins.

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transcriptional modulation of genes implicated in various cellular functions.

In the present study, we reported the characterization of two NRF-1 sites spaced 16 bp apart within the *E2F6* core promoter at positions $-11/+1$ and $+18/+29$ that are essential for basal promoter activity. These sites, close to the major transcription start sites, are conserved in the promoters of human, mouse and rat *E2F6* genes. DNase I footprinting analysis and EMSA (electrophoretic mobility-shift assay) revealed that these sites are bound by NRF-1 present in nuclear extracts, and we demonstrated by ChIP that NRF-1 was associated with the regulatory region of the *E2F6* gene *in vivo*. Moreover, integrity of both sites was found to be critical for efficient basal transcription of the *E2F6* gene, as well as for NRF-1 activation or DN-NRF-1 (dominant-negative NRF-1) repression of *E2F6*.

EXPERIMENTAL

Cell culture

Human bone osteosarcoma U2OS (A.T.C.C. number HTB-96) and mammary gland adenocarcinoma MDA-MB-468 (A.T.C.C. number HTB-132) cell cultures were routinely maintained in DMEM (Dulbecco's modified Eagle's medium) with 10 % fetal calf serum and kept at 37 °C in a water-saturated 5 % CO₂ atmosphere.

DNase I footprinting

DNase I footprinting was performed using the Core Footprint kit according to manufacturer's recommendations (Promega). The -62 to $+103$ *E2F6* promoter region subcloned in the PGL3-basic vector (Promega) was linearized by *HindIII* or *Asp718* digestion and labelled with [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs). The single end-labelled DNA was then digested with *Asp718* or *HindIII* and the 220-bp fragment purified on a native 6 % polyacrylamide non-denaturing gel. The DNase I protection assays were performed using 30 000 cpm of labelled probe in a 50 μ l volume of binding reaction, containing 1 μ g of poly[d(I-C)] and 10 μ g of MDA-MB-468 nuclear extracts prepared according to the method of Dignam et al. [25]. After a 30-min incubation on ice, 50 μ l of a solution (room temperature -22 °C) of 5 mM CaCl₂ and 10 mM MgCl₂ was added to each reaction and incubated for 2 min at room temperature. DNase I (3 μ l, 100 ng/ml) was then added and incubated for 3 min at room temperature. DNase I digestion was stopped by the addition of 90 μ l of stop solution (200 mM NaCl, 30 mM EDTA and 1 % SDS), and the DNA template was purified by phenol/chloroform (1:1) extraction and ethanol precipitation. Samples were analysed by electrophoresis through denaturing 6 % polyacrylamide/urea sequencing gels with a 25 bp DNA ladder (Invitrogen) and an M13 forward sequencing reaction as a ladder.

Plasmid constructions

Mutant *E2F6* promoters were constructed from the parent plasmids ($-1614/+290$)pGL3 and ($-31/+103$)pGL3 [15] using the QuikChange site-directed mutagenesis kit (Stratagene) and the oligonucleotides F6($-31/+8$)Mut1, 5'-TGGGAGCGCTCCGGCAGCGGCGGTTCATTTCGACAGAGGGGG-3', and F6($+9/+38$)Mut2, 5'-GCGGTGTACTGCTTCATTGCGGAAGATGGCG-3'.

NRF-1 and DN-NRF-1 expression vectors were constructed by PCR with *Pfu* polymerase (Stratagene) using the following oligonucleotides: 5'-FLAG NRF, 5'-GATCAGATCTGAGGAACACGGAGTGACCCAAACCGAAC-3'; 3'-NRF, 5'-GATCTCTA-

GATTACTGTTCCAATGTCACCACCTCCAC-3'; and 3'-DN-NRF, 5'-GATCTCTAGATTAGACTACAGTCTGTGATGGTACAAGATG-3'. The *BglIII/XbaI*-digested PCR products were then ligated into the *BamHI/XbaI* sites of the pcDNA3.2-FLAG-N-term vector (Invitrogen) to obtain the plasmids pcDNA3-FLAG-NRF-1 and pcDNA3-FLAG-DN-NRF-1. All constructs were verified by sequencing.

Multiple alignment

Multiple alignments were performed using ClustalW (<http://www.infobiogen.fr/>) with genomic sequences for human *E2F6* (GenBank® accession number AY551345), mouse *E2F6* (GenBank® accession number AF393244) and rat *E2F6* (LocusLink number 313978).

Transient transfections and promoter activity assays

Transfections were performed by the Ex-Gen 500 (Euromedex) procedure in 12-well plates. For co-transfection, 400 ng of reporter plasmid were transfected with increasing amounts of the NRF-1- or DN-NRF-1-expressing plasmids, supplemented with appropriate quantities of the empty vector to maintain constant amount of DNA. Cells were harvested 24 h later and luciferase assays were performed (Promega). All the assays were normalized to the β -galactosidase activity of a CMV (cytomegalovirus)- β -galactosidase vector (25 ng). Experiments were performed at least twice using two different plasmid preparations.

EMSAs

EMSAs were performed using IvT (*in vitro*-translated) proteins, total extracts of U2OS transfected cells or MDA-MB-468 nuclear extracts, as described previously [26]. Briefly, extracts were incubated for 20 min in a final volume of 20 μ l containing 20 mM Hepes, pH 7.9, 20 % glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 3 μ g of sheared salmon sperm DNA, 50 mM NaCl and 50 000 cpm of probe. The reactions were then loaded on to a 5 % polyacrylamide gel in 0.5 $\times$ TBE (Tris/borate/EDTA) and run for 4 h at 170 V (at 4 °C). When indicated, a 200-fold molar excess of unlabelled double-stranded oligonucleotides was added. The following oligonucleotides were employed in binding assays after hybridization to obtain the corresponding DNA duplex: NRF-1cons5', 5'-ATGCTAGCCCCGATGCGCGCGCACCTT-3' (mutated version: TTA) and NRF-1cons3', 5'-AAGGTGCGCGCGCATGCGGGCTAGCAT-3'; F6($-31/+8$)5', 5'-TGGGAGCGCTCCGGCAGCGGCGGATGCGCAGAGGGGG-3' (mutated version: TCATT) and F6($-31/+8$)3', 5'-CCCCCTCTGCGCATGCCCCGCCGTGCCGAGCGCTCCCA-3'; F6($+9/+38$)5', 5'-GCGGTGTACTGCGCATGCGGAAGATGGCG-3' (mutated version: TCATT) and F6($+9/+38$)3', 5'-CGCCATCTTCCGCGATGCGCAGTACACCGC-3'; MYCcons 5', 5'-CCCCACCAGTGGTGCCTGACACGTG-3' (mutated version: TTGA) and MYCcons 3', 5'-CACGTGTCAGGCACCACTGGTGGGG-3'; SP1cons 5', 5'-ATTCGATCGGGGCGGGGCGAGC-3' (mutated version: TT) and SP1cons 3', 5'-GCTCGCCCCGCCGATCGAAT-3'; E2Fcons 5', 5'-ATTAAAGTTTCGCGCCCTTTCTCAA-3' (mutated version: AT) and E2Fcons 3', 5'-TTGAGAAAGGGCGCGAAACTTAAAT-3'. Anti-FLAG-M2 (1 μ l; Sigma) or 2 μ l of anti-(cytochrome *c*) (PharMingen), as a non-specific antibody, were added to binding reactions for super-shift experiments.

ChIP assays

In vivo detection of *E2F6* promoter-associated NRF-1 was performed by ChIP, as described previously [26], on U2OS cells

Figure 1 Sequence alignment of the human, mouse and rat *E2F6* promoter

The human *E2F6* sequence (200 bp) surrounding the transcription start site was aligned with the mouse and rat *E2F6* corresponding sequences using the ClustalW program. Identical nucleotides are indicated by asterisks under the sequences. The numbers are relative to the transcription start sites determined for the human (indicated by a white arrowhead) and mouse (indicated by a black arrowhead) gene [14,15]. The putative NRF-1-binding sites are shaded.

overexpressing the FLAG-NRF-1 protein. The FLAG-NRF-1-containing complexes were immunoprecipitated with a polyclonal anti-FLAG antibody (Santa Cruz, D-8), as well as with a pool M2 and M5 anti-FLAG monoclonal antibodies (Sigma-Aldrich). The anti-DBD-Gal4 (DNA-binding domain of Gal4) monoclonal RK5C1 antibody (Santa Cruz) and the anti-ICAM-1 (intercellular cell-adhesion molecule 1) M-19 polyclonal antibody (Santa Cruz) were used to control the immunoprecipitation specificity. The following oligonucleotides were designed to amplify by PCR a 150 bp fragment encompassing the $-39/+111$ region of the human *E2F6* promoter: 5'E2F6p, 5'-TCGGTGCCTGGGAGCG-CTCCGGC-3', and 3'E2F6p, 5'-CTCACGTGCCCGGGAGC-TCCCGAC-3'. Specific primers were also designed to amplify a 146-bp fragment of the sixth exon of the human *E2F6* gene as a negative control: 5'E2F6ex6, 5'-CTTTGTCACTCTGTTAACTC-3', and 3'E2F6ex6, 5'-TCTGATCTTAGCAATTTTGG-3'. Finally, specific primers were designed to amplify a 146-bp fragment of the human *FMRI* (fragile X mental retardation 1) promoter as a positive control: 5'FMRIp, 5'-CGAGGCAGTGCGACCTGTC-AC-3', and 3'FMRIp, 5'-CTCTTCAAGTGGCCTGGGAGC-3'.

RESULTS

DNase I footprinting analysis of the *E2F6* proximal promoter reveals protection of two potential NRF-1 sites

We previously published the initial characterization of human and mouse *E2F6* promoter [14,15]. Transient transfection studies allowed us to map a 134 bp human core promoter fragment located between -31 and $+103$ and a 130 bp mouse core promoter fragment located between -55 and $+75$ with regards to the transcription start site. These two GC-rich promoter regions devoid of TATA box or CAAT box led to multiple transcriptional start sites [14,15]. A comparison of the 200 bp promoter region surrounding the transcriptional start site of the human and mouse genes with the sequence of the rat *E2F6* gene found in the databank (LocusLink 313978) reveals a high degree of overall sequence conservation (60% identity) along with several short stretches of absolute identity (Figure 1). Among them, the comparison allow us to identify putative NRF-1 sites with core sequences perfectly conserved. In order to determine if the phylogenetic conservation of the NRF-1 sites is consistent with a functional role in controlling *E2F6* expression, we analysed the DNA-protein interactions of the minimal human *E2F6* promoter by DNase I footprint analysis. Nuclear extracts from MDA-MB-468 cells were tested for their ability to protect end-labelled DNA promoter fragment

(-31 to $+103$) from digestion with DNase I. As shown in Figure 2(A), four distinct regions (named FPI–FPIV) were found to be protected in a reproducible manner on both the sense and antisense strands by proteins present in the extracts.

Scanning of several databases for transcription factor binding motifs matching with the observed protected regions was negative. However, two protected regions, FPI and FPIII partially overlapped sequences which share obvious sequence similarity with the NRF-1 transcription factor binding site. The optimal NRF-1 binding site is (T/C)GCGCA(C/T)GCGC(A/G) [27], whereas the human *E2F6* promoter harbours the elements CGGGCATGCGCA (nucleotides -11 to $+1$) and TGCATGCGGG (nucleotides $+18$ to $+29$). The results showed that bases -11 to -5 of the more upstream putative NRF-1 binding site were protected against DNase I digestion on the coding strand, whereas the -5 to $+1$ residues were protected on the complementary strand. On the other hand, the $+20$ to $+24$ residues of the second putative NRF-1 binding site were protected against DNase I digestion on both the coding and the non-coding strands, whereas the $+25$ to $+29$ residues of the site were protected only on the coding strand (Figure 2B).

NRF-1 binds NRF-1-binding sites on the human *E2F6* promoter

To demonstrate that the DNase I footprint of the FPI and FPIII regions resulted from NRF-1 binding, we performed EMSA experiments. Double-stranded oligonucleotides spanning each putative NRF-1 binding site were therefore synthesized and radiolabelled. EMSAs were performed with IvT human recombinant FLAG-NRF-1 proteins. As expected, a major band of a protein–DNA complex was observed when the IvT NRF-1 proteins were incubated with the $-31/+8$ and the $+9/+38$ probes (Figure 3A). In both cases, the complex was competed by a 200-fold molar excess of an unlabelled double-stranded oligonucleotide containing the functional NRF-1 consensus site from the rat somatic cytochrome *c* promoter (-173 to -147) [17], but not by an oligonucleotide containing a mutated NRF-1 binding site. The binding on each probe of the IvT NRF-1 proteins was unaffected by competition with a non-specific consensus binding site oligonucleotide (E-box). Moreover, addition of anti-FLAG antibodies diminished the initial complex formation and led to the apparition of a supershifted complex, thus confirming the presence of FLAG-NRF1 in the complexes. Non-specific IvT E2F6–DP1 proteins were also added to confirm the specificity of the observed DNA–NRF-1 complex. So, these results indicate that the two putative NRF-1 binding sites present in the *E2F6* core promoter can recruit NRF-1 proteins.

To further demonstrate that these sequences are effectively recognized by cellular NRF-1 proteins, we performed EMSA with MDA-MB-468 and U2OS cell nuclear extracts. Similar results were obtained with both cell extracts and results presented thereafter are those obtained with U2OS cell nuclear extracts. As shown in Figure 3(B) (left panel), incubation of the (-31 to $+8$) probe with the U2OS cell nuclear extracts resulted in the formation of two distinct protein–DNA complexes. The slower migrating complex was eliminated by addition of a 200-fold excess of unlabelled oligonucleotides containing the rat cytochrome *c* NRF-1 consensus site or *E2F6* $-31/+8$ probe, but not by a mutated version of these sites. Moreover, incubation of U2OS cell nuclear extracts with mutated $-31/+8$ probe, in which the core CATG of the NRF-1 site was replaced by AATT, results in a complete loss of the slower migrating complex, suggesting that the latter is a genuine NRF-1 complex. On the other hand, the faster migrating complex seems to be non-specific, since it was strongly competed by either the wild-type and the mutated NRF-1 unlabelled

Figure 2 Identification of protein-binding sites in the human *E2F6* promoter by DNase I footprinting

(A) The sense (Asp718 probe) and the antisense (HindIII probe) strands of the human *E2F6* promoter in the region spanning the nucleotides from -31 to $+103$ relative to the transcription start site were analysed by DNase I footprinting with MBA-MD-468 cell nuclear extracts (NE). The nucleotides that were found to be protected on both strands are indicated by the boxes, with their relative positions with respect to the transcription start site indicated. The DNase I hypersensitive sites are indicated with asterisks. Lanes corresponding to probes digested with DNase I were duplicated for better visualization of footprints. (B) The identified protein-binding regions are mapped on to the DNA sequence with footprints obtained in sense (shaded grey) and antisense (boxed) orientations.

oligonucleotides, and was unaffected by mutation of the NRF-1 site on the $-31/+8$ probe. Similarly, incubation of the $+9/+38$ probe with nuclear extracts yields two protein–DNA complexes (Figure 3B, right panel). Competition with a 200-fold excess of intact, as well as mutated, NRF-binding site failed to abolish the faster migrating complex, indicating that it is probably a non-specific complex. By contrast, the slower migrating complex contains NRF-1 proteins, whereas its formation is prevented by the addition of an unlabelled wild-type NRF-1 oligonucleotide, but is unaffected by the mutated one. Moreover, the binding is lost when the NRF-1 binding site was mutated in the $+9/+38$ probe.

The NRF-1 binding sequence in the *E2F6* FPIII region (included in the $+9/+38$ probe) overlapped in part with an E2F binding site (Figure 2B). Moreover, both NRF-1 binding sequences present in the *E2F6* promoter regions $-31/+8$ and $+9/+38$ in-

clude a non-canonical Myc–Max-binding site [CA(C/T)GCG] that may be targeted by c-Myc, as demonstrated for the cytochrome *c* promoter [28]. In order to determine whether the complexes observed with nuclear extracts on the *E2F6* probes contain these transcription factors, competition experiments were performed with consensus E2F, E-box or SP1 unlabelled oligonucleotides. In fact, none of these consensus oligonucleotides affect the binding observed on the *E2F6*–NRF-1 probes. Therefore, it seems that NRF-1 is the major component of the slower migrating complexes formed with U2OS nuclear extracts on $-31/+8$ and $+9/+38$ probes.

Specificity of NRF-1 binding was further confirmed by super-shift experiments. Total cellular extracts ($1 \mu\text{g}$) from U2OS cells transfected with plasmids encoding the FLAG–NRF-1 protein or the FLAG–DN–NRF-1 [28,29] were tested for their DNA-binding activity on the *E2F6*–NRF-1 probes. As shown in Figure 3(C), this

Figure 3 Binding to *E2F6* promoter elements -31 to +8 and +9 to +38

(A) Gel retardation assay of lvt FLAG-NRF-1 on *E2F6* probes -31/+8 and +9/+38. The NRF-1 protein-DNA complexes are indicated by an arrow. Specificity of the observed NRF-1-containing complexes was assessed by competition experiments with wild-type (WT) or mutated (M) forms of consensus NRF-1-binding sequences as indicated. Asterisks indicated supershift obtained by adding FLAG antibody (α -Flag) to the binding reaction. lvt *E2F6*-DP1 proteins were also added to assess the FLAG-NRF-1-binding specificity. (B) DNA-binding activity of U2OS cell nuclear extracts on *E2F6* promoter. Gel shift analyses were performed with wild-type (WT) or mutated (Mut) probes containing the potential NRF-1-binding sites identified in the -31/+8 and +9/+38 regions of the *E2F6* promoter. The NRF-1 protein-DNA complex is indicated by an arrow. As in (A), unlabelled oligonucleotides were added as indicated to confirm binding specificity. NS, non-specific binding. (C) DNA-binding activity of wild-type NRF-1 protein and dominant negative form (DN) expressed in U2OS cells. Total extract from U2OS transfected with the pcDNA3-FLAG empty vector (A3-FLAG), FLAG-NRF-1 and FLAG-DN-NRF-1 vectors were used in gel retardation assays as in (A). Supershift experiments were performed with the FLAG antibody (α -Flag) and a non-relevant isotype antibody (Control).

quantity of cellular extracts from cells transfected with an empty vector (A3-FLAG) was insufficient to detect the endogenous NRF-1-binding activity, as observed in Figure 3(B). In contrast, extracts from cells expressing FLAG-NRF-1 protein or FLAG-DN-NRF-1 led to the formation of a major binding complex with the -31/+8 probe (Figure 3B, left panel) or the +9/+38 probe

(right panel). The observed complexes were supershifted by the anti-FLAG antibody, but not by the control anti-(cytochrome *c*) antibody assessing their specificity. Taken together, these results confirm that NRF-1 proteins associate with the NRF-1 binding sites on nucleotides -11 to +1 and nucleotides +18 to +29 of the minimal *E2F6* promoter.

NRF-1 transactivates the human *E2F6* promoter

To determine the functional significance of NRF-1 binding on the *E2F6* promoter activity, we next performed transient transfection experiments of U2OS cells. The activity of the wild-type p(−1614/+290) and the minimal p(−31/+103) *E2F6*–luciferase reporter constructs were compared with the activity of their corresponding versions mutated on the (−11/+1)-NRF-1 binding site (M1), on the (+9/+29)-NRF-1 binding site (M2) or on both NRF-1 binding sites (M1+2) (Figure 4A). Results, expressed as fold activity relative to that obtained with the empty pGL3-Basic plasmid, indicated that the M1 and M2 mutations reduced respectively the p(−1614/+290) activity by about 50% and 30%. Mutation of the two sites led only to a 60% reduction in the p(−1614/+290) activity. Interestingly, the introduction of the same mutations in the minimal promoter construct p(−31/+103) resulted in a more pronounced effect on the basal promoter activity, with about 80% decrease in the promoter activity for each single mutation. Likewise, combined mutation resulted in a nearly complete loss of the p(−31/+103) transcriptional activity. These results indicate that NRF-1 binding sites at positions −11 to +1 and +18 to +29 of the *E2F6* promoter are needed for optimal basal activity of the *E2F6* promoter. Nevertheless, the more pronounced effect of mutation of these sites for the p(−31/+103) construct, as compared with p(−1614/+290), may indicate the presence of other important regulatory elements for the promoter activity. Similar results were obtained in Cos-1 and RK-13 cells (results not shown).

To investigate the ability of NRF-1 to potentiate *E2F6* transcription, co-transfections of the NRF-1 expression vector (FLAG–NRF-1) were performed in U2OS cells with the wild-type *E2F6* p(−1614/+290) or p(−31/+103) constructs, as well as with the corresponding mutated promoter constructs. As shown in Figure 4(B), NRF-1 co-transfection induced a 2-fold increase of the p(−1614/+290) and p(−31/+103) activity. In contrast, mutation of the NRF-1 elements in the same constructs completely eliminated the NRF-1 effect.

Further evidence of the requirement of NRF-1 for efficient *E2F6* promoter activity were obtained by co-transfection of the FLAG–DN-NRF-1 expression vector. The p(−1614/+290) and p(−31/+103) constructs promoter activity is dose-dependently inhibited upon expression of FLAG–DN-NRF-1, with around 75% inhibition observed at 5 ng (Figure 4C). Our results clearly indicate that the NRF-1 elements present in the *E2F6* promoter are at least partially responsible for its transcriptional activity and may be implicated in its regulation during physiological processes.

NRF-1 is associated *in vivo* with the *E2F6* promoter

To confirm the involvement of NRF-1 in the *E2F6* promoter control, we performed ChIP experiments upon U2OS cell transfection with the FLAG–NRF-1 expressing vector, as described previously [26]. As shown in Figure 5, the cross-linked *E2F6* promoter–FLAG–NRF-1 complexes immunoprecipitated with either a polyclonal anti-FLAG antibody (D8) or a mix of two monoclonal anti-FLAG antibodies (M2/M5) were detected by PCR amplification with oligonucleotides spanning the −39 to +111 region of the human *E2F6* promoter. As a positive control, *in vivo* association of FLAG–NRF-1 with the *FMRI* promoter, a well-characterized NRF-1 target gene [30], was also detected by PCR with specific primers on the same samples. PCR amplification using primers spanning the sixth exon of *E2F6* gene, which does not possess NRF-1-binding sites, revealed a similar level of non-specific DNA contamination for all the samples, and provides a further control for the specificity of the interaction between

Figure 4 Functional analysis of the human *E2F6* gene promoter

(A) Effect of site specific mutations on human *E2F6* promoter activity in U2OS cells. Site specific mutations were performed on the −1614/+290 *E2F6* promoter fragment and the minimal −31/+103 *E2F6* promoter fragment. The results are expressed as fold normalized activity relative to that obtained with the control pGL3-Basic plasmid (arbitrarily set to 1) and are the means $\pm$ S.D. for at least three independent experiments. All experiments were conducted with pcDNA3- β -galactosidase to normalize the transfection efficiency. On the schematic representation of the promoter, the open circles represent a wild-type binding site, whereas the crossed circles represent a mutated one. (B) Modulation of the *E2F6* promoter activity by exogenous NRF-1 proteins. Increasing amounts of expression vector encoding FLAG-tagged NRF-1 were co-transfected with 0.4 μ g of wild-type or mutated *E2F6* (−1614/+290) promoter reporter constructs (upper panel) or wild-type or mutated *E2F6* (−31/+103) promoter reporter constructs (lower panel). The results are presented, for each promoter construct, as the fold increase in normalized luciferase activity relative to that obtained with the wild-type promoter with the empty pcDNA3-FLAG expression plasmid (arbitrarily set to 100). (C) Increasing amounts of expression vector encoding FLAG–DN-NRF-1 were co-transfected with 0.4 μ g of wild-type or mutated *E2F6* (−1614/+290) promoter reporter constructs (upper panel) or wild-type or mutated *E2F6* (−31/+103) promoter reporter constructs (lower panel). The activities of the different constructs are presented as in (B).

Figure 5 NRF-1 association with the *E2F6* promoter *in vivo*

Formaldehyde cross-linked chromatin from transfected U2OS cells with the FLAG-NRF-1 expression vector were subjected to ChIP experiments. Immunoprecipitations were performed using polyclonal (D8) or monoclonal (M2/M5) antibodies directed against the FLAG tag. Anti-ICAM-1 (D8) polyclonal antibodies and the anti-DBD-Gal4 monoclonal antibodies were used as negative controls. After isolation of bound DNA, PCR was performed for a 150 bp region of the endogenous *E2F6* promoter. PCR reactions were also performed using *FMR1* promoter-specific primers, a well known NRF-1 target gene, as a positive control, and *E2F6* exon 6-specific primers as a negative control. For each experiment, the number of PCR cycles in indicated. Input indicates PCR performed on DNA (1/400) without any immunoprecipitation.

NRF-1 and the *E2F6* promoter. As a negative control, ChIP with non-specific antibodies directed against ICAM-1 or DBD-Gal4 resulted in the absence of signal for the *E2F6* promoter, as well as for *FMR1*. These results demonstrated that NRF-1 binds the *E2F6* promoter *in vivo* and provide further evidence for NRF-1 transcriptional control of *E2F6* promoter activity.

DISCUSSION

We previously defined the murine and human *E2F6* promoter region [14,15]. The present study has extended these previous results. Alignment of the mouse and human *E2F6* core promoter sequences reveals a 66% identity within the 200 nt surrounding the start sites and 85% identity between the murine and rat sequences. DNase I footprint analysis was performed to localize the major regulatory elements present in the *E2F6* proximal promoter region. Four major protected areas were identified (FPI–FPIV). Computer-based analysis of these sequences identified two potential binding sites for NRF-1 (FPI and FPIII), which differ from the optimal consensus NRF-1 binding site (T/C)GCGCA(C/T)GCGC(A/G) [27] by 1 bp. Although the perfect consensus is probably the best NRF-1 binding site, a large number of binding sequences in genes known to be regulated by NRF-1 revealed a more general consensus. The presence of other protected regions (FPII and FPIV) indicates additional protein–DNA interactions with the human *E2F6* core promoter region. No clear similarity was found between these sequences and any genuine consensus binding sites, and further studies are required to determine which proteins could be implicated. Nevertheless, it should also be noted that these sequences are poorly conserved in the other mammalian genes, indicating probably a more marginal effect on *E2F6* regulation. Conversely, the two NRF-1 sites are well conserved among human, murine and rat *E2F6* core promoters, supporting the hypothesis that they play an essential role in *E2F6* gene regulation. As expected, EMSA assays performed with mouse *E2F6* probes, encompassing the potential NRF-1 binding sites (–37 to +1 and +2 to +39 relative to the transcription start site), showed NRF-1 binding to these sites (results not shown). With regard to the human gene, these two NRF-1 elements seem to be essential for optimal *E2F6* gene ex-

pression, since their mutation dramatically decreased the promoter activity in U2OS cells. Nevertheless, a more pronounced mutation effect was observed for the p(–31/+103) construct than for p(–1614/+290), suggesting that other regulatory elements are localized between nucleotides –1614 and –31. Interestingly, overexpression of NRF-1 activated both *E2F6* promoter constructs. Although the overall NRF-1 effect on *E2F6* transcription is relatively low, it is in the range of that observed for the rat Tfam promoter and the human eIF-2 α (eukaryotic translation-initiation factor-2 α) promoter, two well-known NRF-1 target genes [16,31]. Moreover, *E2F6* promoter activity is completely abolished upon transfection of DN-NRF-1, as previously described for other NRF-1 target genes [28,29]. The potential involvement of NRF-1 in the control of the *E2F6* transcriptional activity is also reinforced by the fact that NRF-1 is associated *in vivo* with the *E2F6* promoter as demonstrated by ChIP.

Therefore, these results indicate that endogenous NRF-1 is important for efficient *E2F6* promoter activity. Interestingly, a large number of NRF-1 target genes, including *E2F6*, present common features, such as multiple transcription start sites, absence of canonical TATA or CCAAT boxes and GC-rich content [14,15,22,32]. Moreover, most of these genes encode products that are widely expressed. This is in agreement with the wide distribution of *E2F6* that correlates well with NRF-1. In adult tissues, these genes are ubiquitously co-expressed in all tissues analysed and their highest expression is found in skeletal muscle [7,14,27,33]. Moreover, the NRF-1 gene was expressed during oogenesis and during early stages of embryogenesis [19]. Similarly, Northern blot experiments performed on total RNA from various stages of mouse embryonic development revealed *E2F6* expression at a high level from day 9 to day 18 post-coitum [14], whereas *in situ* hybridization showed a broad pattern of *E2F6* expression at low level on E14.5 embryos [12].

The best defined biological role for NRF-1 is in the nuclear control of mitochondrial respiratory function [34]. In addition, NRF-1 has also been implicated in other cellular functions. Efioik and Safer [35] demonstrated that overexpression of NRF-1 increased both protein synthesis and growth by upregulating the transcription of eIF-2 α and eIF-2 β genes, but retarded cell cycle progression by the repression of E2F1 gene transcription. Moreover, NRF-1 sites are found in genes that may be directly involved in cell cycle regulation (cdc2 and the guanine-nucleotide exchange factor RCC1) or are regulated by cell growth [DNA polymerase- α , GADD153 (growth-arrest and DNA-damage-inducible protein 153) and ornithine decarboxylase] [27]. Nevertheless, despite a growing number of publications and potential implication of NRF-1 as a key regulator of numerous crucial cellular processes, nothing is really known concerning the regulation of its activity, except a potential enhanced NRF-1 DNA-binding capacity upon phosphorylation [36,37].

Our finding that NRF-1 directly regulates *E2F6* complements the published observation that NRF-1 may participate in coordinating the regulation of global protein synthesis, growth and cell cycle [27,35]. To date, our results only suggest a role for NRF-1 in the control of basal *E2F6* transcription and may partially explain its wide expression in tissues and cell lines. Analysis of *E2F6* expression in *NRF-1*^{–/–} mice would have been very interesting, but these animals died between embryonic days 3.5 and 6.5, and their blastocysts are unable to grow *in vitro* [19]. Consistent with these observations, NRF-1 belongs to a family of developmentally expressed transcription factors. Invertebrate NRF-1 homologues, P3A2 [38] and EWG (erect wing gene product) [39], as well as the zebrafish (*Danio rerio*) homologue *nrf* (not really finished) [40], have been implicated in embryonic or larval development. Interestingly, *E2F6* knock-out experiments have revealed that

E2F6 protein is critical for developmental patterning [12]. Our results raises the possibility that the NRF-1 transcription factor could act, partially via *E2F6*, in human developmental patterning, similar to its invertebrate homologues.

We thank J. L. Baert for critical reading of the manuscript. This work has been carried out on the basis of grants awarded in part by the Centre National de la Recherche Scientifique, the Institut Pasteur de Lille, the Université des sciences et technologies de Lille 1, the Association pour la Recherche sur le Cancer and the Ligue Contre le Cancer (France).

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Received 3 June 2004/12 July 2004; accepted 19 July 2004

Published as BJ Immediate Publication 19 July 2004, DOI 10.1042/BJ20040935