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8-Chloro-Adenosine-Induced E2F1 Promotes *p14ARF* Gene Activation in H1299 Cells Through Displacing Sp1 From Multiple Overlapping E2F1/Sp1 Sites

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ABSTRACT

The regulation of *p14ARF* gene by E2F transcription factor, which differs from that of classical E2F targets, has recently been attributed to a variant E2F-response element. However, promoter assays suggest multiple elements present in the *p14ARF* promoter and argue against the idea that the *ARF* promoter has a unique ability to distinguish between aberrant and physiological levels of E2F1. Therefore, the functional characterization of the promoter still needs to be done. We demonstrate that at least two overlapping E2F1/Sp1 binding sites are present in the *p14ARF* promoter, and E2F1 activates the promoter through displacing constitutive Sp1 from the overlapping sites. We found that 8-chloro-adenosine (a metabolite of 8-Cl-cAMP) exposure induced the *p14ARF* gene in human lung cancer H1299 cells, followed by increased expression of E2F1 and constitutive expression of Sp1. The combination of cotransfection and electrophoretic mobility shift assay (EMSA) indicated that constitutive binding of Sp1 to the overlapping sites contributed to a constitutive expression of the *ARF* gene in unexposed H1299, whereas displacing Sp1 from the overlapping sites by E2F1 promoted the gene activation after exposure. EMSA and chromatin immunoprecipitation revealed increased association of E2F1 with the overlapping sites in the active promoter in 8-Cl-Ado-exposed cells. Together, these data suggest that the overlapping E2F1/Sp1 site, being present in multiple copies in the *p14ARF* promoter, may serve as the targets for both E2F1 and Sp1, thereby playing a crucial role in response to some oncogenic signals and stimulators, which activate the *ARF* gene through inducing E2F in the cell. J. Cell. Biochem. 106: 464–472, 2009.

KEY WORDS: p14ARF; E2F1; SP1; GENE REGULATION; OVERLAPPING E2F1/Sp1 SITE; COMPETITIVE DISPLACEMENT; 8-CHLORO-ADENOSINE

Human tumor suppressor p14ARF (p19ARF in mouse) is an alternative transcript of the INK4a-ARF tumor suppressor locus that encodes the p16INK4a suppressor [Sherr, 2001]. Two products of the locus are transcriptionally controlled by separate promoters [Haber, 1997]. The ARF suppressor inhibits cell growth by inhibiting MDM2 and stabilizing p53, eliciting G1 and G2 cell-cycle arrest [Quelle et al., 1995; Stott et al., 1998]. In addition, ARF is involved in apoptosis [Zindy et al., 1998] and senescence [Dimri et al., 2000; Phillips and Vousden, 2001; Randle et al., 2001].

The *p14ARF* promoter is a CpG island characteristic of a housekeeping gene [Robertson and Jones, 1998]. Although the *p14ARF* gene can be silenced by de novo methylation within CpG islands, it is a limited event in cell lines [Robertson and Jones, 1998; Fukai et al., 2005]. The *ARF* gene can be induced by oncoproteins, in which at least some oncogenic signals act through activating E2F-dependent transcription [DeGregori et al., 1997; Bates et al., 1998]. Recently, the regulation of *p14ARF* gene by E2F, which differs from that of classical E2F targets, has been attributed to a variant E2F-response element (termed the EREA) [Komori et al., 2005]. However, attempts to search *cis*-elements cannot find critical E2F regulatory motifs [del Arroyo et al., 2007] and reveal multiple-response elements present in the core promoter [Robertson and Jones, 1998; Parisi et al., 2002; del Arroyo et al., 2007]. The *p14ARF* promoter contains numerous Sp1 binding sites and E2F sites [Robertson and

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Jones, 1998]. Overexpression of E2F1 [Robertson and Jones, 1998; Parisi et al., 2002; Lowe and Sherr, 2003; Komori et al., 2005] can activate the *p14ARF* promoter. It is suggested that through binding to multiple elements with low affinity, E2F1 might act in conjunction with other factors such as Sp1 [del Arroyo et al., 2007]. However, how Sp1 and E2F1 cooperate to regulate the gene is unclear, and the functional characterization of the promoter still needs to be done.

Based on sequencing [Robertson and Jones, 1998], we found four putative E2F1 binding sites overlapping with the Sp1 site in the *p14ARF* promoter (Table I). We infer that the overlapping E2F1/Sp1 sites might be crucial for coordinate regulation of the *p14ARF* gene activation by E2F1 and Sp1. We have described that 8-chloro-adenosine (8-Cl-Ado), a metabolite of 8-Cl-cAMP (anti-cancer drug), can induce DNA double-stranded breaks [Yang et al., in press] and G2/M arrest [Zhang et al., 2004; Gu et al., 2006; Jia et al., in press] in cancer cells, followed by apoptosis [Zhang et al., 2004; Gu et al., 2006]. Recently, we found that 8-Cl-Ado-induced apoptosis is associated with the *p14ARF* upregulation (unpublished data). Herein, we demonstrate that the overlapping E2F1/Sp1 site, being present in multiple copies in the *p14ARF* promoter, can serve as the targets for both E2F1 and Sp1: binding of Sp1 to the overlapping sites resists E2F1-promoted ARF activation, maintaining a constitutive expression in H1299 cancer cells, whereas displacing of Sp1 at the overlapping sites by increased E2F1 promotes the ARF activation in response to 8-Cl-Ado. These data suggest that E2F1 and Sp1 cooperate to regulate the *p14ARF* gene expression through a competitive displacement mechanism.

MATERIALS AND METHODS

CELL CULTURE AND CHEMICAL TREATMENT

Human lung cancer H1299 cell (ATCC, Rockville, MD) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown at 37°C with 5% CO_2. 8-Cl-Ado (State Laboratory for Natural and Biomimetic Drugs, Peking University, China) exposure was performed as described [Gu et al., 2006].

WESTERN BLOTTING

As described [Zhang et al., 2004], 30 μg proteins was subjected to SDS–PAGE, transferred onto nitrocellulose membranes, and probed with specific antibodies (1:1,000) for p14ARF (Chemicon International, Temecula, CA), E2F1, Sp1, or Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed with a secondary antibody (IgG)-conjugated horseradish peroxidase. Chemiluminescence signals were visualized using Western blotting luminol reagent (Santa Cruz Biotechnology).

TABLE I. The Overlapping E2F1/Sp1 Sites in the *p14ARF* Promoter

Position	Sequence
−275/−265	AGGGCGGGAAA
−257/−249	TAGGCGGGA
−76/−68	AAGGCGGGT
+27/+35	AAAGGGCGG

E2F1 binding site underlined.

QUANTITATIVE REAL-TIME PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of RNAs was subjected to reverse transcription by M-MLV reverse transcriptase (Promega, Madison, WI). PCR amplification was performed in triplicate using the following primers: for human GAPDH, (sense) 5′-TGTCAGTGGTG-GACCTGACCT-3′ and (antisense) 5′-AGGGGAGATTCAGTGTGGTG-3′; and for p14ARF, (sense) 5′-GGTTTTCGTGGTTCACATCCCGCG-3′ and (antisense) 5′-CAGGAAGCCCTCCCGGGCAGC-3′. Each PCR mixture contained 5 μl of cDNA, 400 nM concentration of each primer, and 25 μl of SYBR® Green Real-Time PCR Master Mix (TOYOBO, Osaka, Japan) in a 50 μl reaction mixture. Real-time PCR was performed using ABI PRISM® 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). The quantity of *p14ARF* mRNA (C_t of p14ARF) was normalized by subtracting the quantity of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (C_t of GAPDH) (internal control) to obtain a normalized value of $\Delta C_{t\text{-}p14ARF}$. A $\Delta\Delta C_t$ value was deduced by subtracting the exposed $\Delta C_{t\text{-}p14ARF}$ by the unexposed $\Delta C_{t\text{-}p14ARF}$. The relative quantity of *p14ARF* mRNA was obtained using the value of $2^{-\Delta\Delta C_t}$, according to the manufacturer's protocol.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

ChIP was performed following the protocol provided in anti-acetyl-histone H4 ChIP assay kit (Upstate, NY). Cells were incubated in 1% formaldehyde at 37°C for 10 min, then lysed and sonicated to shear DNA to 200–1,000 bp in length. Subsequently, 5 μl of anti-acetyl-histone H4 antibody (Upstate), 1 μg of anti-E2F1 (C-20) or 1 μg of anti-Sp1 (PEP 2) antibody (Santa Cruz Biotechnology) were added for immunoprecipitating the chromatin. DNA samples were purified using Wizard® PCR Preps DNA Purification System (Promega). Regular PCR and real-time PCR amplification were performed using specific primers for the *p14ARF* promoter regions from −281 to −56 (P1 and P2) (Fig. 3A). Input of every sample was used as an internal control.

CONSTRUCTS AND TRANSFECTION

pGL3ARF(−735/+54) was provided by Gordon Peters. To generate mutant plasmid, pairs of synthesized oligonucleotides were used as primers: for −735/−243 fragment, (sense) 5′-ATCGATAGGTA-CCGGGCCCCCCCTC-3′ and (antisense mutant) 5′-TCTCCCTCCCG-CCTACCGCCACgggtCgaCCCTGTGTGC-3′; for −281/+54 fragment (sense mutant) 5′-GCACACAGGGtcGacccGTGGCGGTAGGCGG-GAGGGAGA-3′ and (antisense) 5′-GGCTAGCACGCGTAAGAGC-TCGGCA-3′. PCR products were mixed as templates for next PCR, generating a −735/+54 fragment mutated at the −275/−265 overlapping E2F1/Sp1 site. The fragment was inserted into *Kpn*I/*Sac*I sites of pGL3-Basic to obtain pGL3ARF(−735/+54)m. Transfection was performed by Lipofectamine™ 2000 (Invitrogen) according to manufacturer's protocol.

ENZYME ASSAY

Firefly and *Renilla* luciferase activities were assayed using Dual-Luciferase® Reporter Assay System (Promega). *Firefly* luciferase activities were verified for transfection efficiencies as computed relative to *Renilla* luciferase activities.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts (NE) were prepared as described [Jia et al., 1992]. GST-E2F1 fusion protein was expressed by pGEX20T-E2F1 (a gift from Kristian Helin) in bacteria and purified using Glutathione Sepharose 4B (Amersham Biosciences, Uppsala, Sweden). EMSA was performed as follows. Prior to the addition of biotin-labeled probe, 2 μg NE or varied amount of GST-E2F1 and/or rhSp1 (Promega) was incubated for 10 min at room temperature in binding buffer. Then 2 μl (20 fmol) of probe was added. In competition experiments, 200× M excess of unlabeled oligonucleotides were used. In super-shift experiments, anti-E2F1 (C-20) or anti-Sp1 (PEP 2) antibodies (1 or 2 μg; Santa Cruz Biotechnology) were added. Protein–DNA complexes were separated on 6% PAGE and visualized by LightShift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL).

RESULTS

8-Cl-ADO EXPOSURE UPREGULATES E2F1-ACTIVATED p14ARF IN VIVO

In H1299 cells (*p14ARF*-wt, *p53*-null), the p14ARF protein (Fig. 1A) and mRNA (Fig. 1B) were constitutively expressed at low levels. In 8-Cl-Ado-exposed cells, however, the protein and mRNA were upregulated within 48–96 h after exposure, indicating the activation of the *ARF* gene during 8-Cl-Ado exposure. Notably, the E2F1 protein was also increased (Fig. 2A), in parallel with upregulated p14ARF protein. However, Sp1 was constitutively expressed at relatively abundant levels before and after exposure (Fig. 2B).

To test whether the upregulated E2F1 is associated with the *p14ARF* promoter activation, ChIP was performed using anti-acetyl-histone H4, anti-E2F1, and anti-Sp1 antibodies, respectively, followed by regular PCR (Fig. 2C, left panel) and real-time PCR (Fig. 2C, right panel) amplification of the −281/−56 region in the *p14ARF* promoter. ChIP assays showed increased E2F1 binding to the promoter in 8-Cl-Ado-exposed H1299. Whereas, Sp1 binding to the promoter was little changed before and after exposure. These results indicate that induction of E2F1 by 8-Cl-Ado is correlated to the *ARF* gene activation.

DISTAL OVERLAPPING E2F1/Sp1 SITE IS NECESSARY BUT NOT SUFFICIENT FOR FULL PROMOTER ACTIVITY

To test the activation of the p14ARF gene by 8-Cl-Ado, we transfected the reporter construct pGL3ARF(−735/+54), containing the *Luc* gene under the control of the *p14ARF* promoter, into H1299, and exposed the cells to 8-Cl-Ado. Luciferase activities showed that the pGL3ARF(−735/+54) reporter increased 0.8- to 2.3-fold of a basal activity (0 h) after 72–96 h exposure (Fig. 3B), indicating that 8-Cl-Ado does promote the *p14ARF* promoter activity.

The *p14ARF* promoter contains four E2F1 sites overlapping with the Sp1 site and seven simple Sp1 sites [Robertson and Jones, 1998]. To investigate the role of overlapping E2F1/Sp1 binding site in the promoter activation, we mutated the distal overlapping site at position −275/−265 (Fig. 3A and Table I) but kept downstream overlapping sites to construct a mutant promoter-driven reporter pGL3ARF(−735/+54)m. Transfection and reporter enzyme activities (Fig. 3C) showed that in unexposed H1299 (0 h), the enzyme activities expressed by wild-type and mutant reporters had no significant difference. However, in 72 h exposed cells, the wild-type construct increased 113% of the unexposed activity, while the mutant increased only 66% ($P < 0.05$, wt vs. mut.). These results indicate that the −275/−265 overlapping site is responsible at least in part for 8-Cl-Ado-upregulated ARF. These data also suggest that the distal overlapping site is necessary but not sufficient for full promoter activity.

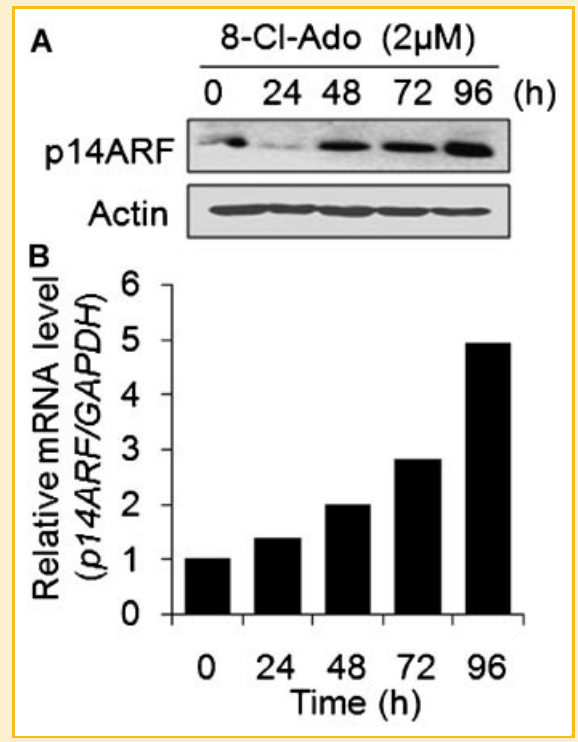

Fig. 1. Upregulation of p14ARF protein and mRNA by 8-Cl-Ado exposure. H1299 cells were exposed to 8-Cl-Ado (2 μM) for given hours. After harvest, cells were lysed, and (A) proteins were quantified for Western blot analysis of p14ARF. Actin as a loading control. B: RNAs were analyzed by real-time PCR (see Materials and Methods Section). GAPDH as an internal control.

Sp1 DOMINANTLY BINDS TO OVERLAPPING SITES BEFORE EXPOSURE

To determine the binding of E2F1 and Sp1 to the overlapping E2F1/Sp1 sites, we performed EMSA using an oligonucleotide as probe, representing −281/−243 region which bears two putative overlapping sites (Table I, Figs. 3A and 4A). Seven (A–G) protein–DNA complexes occurred, when the probe was incubated with unexposed NE (Fig. 4B, lane 2). Interestingly, similar pattern with complexes B–G was detected, except complex A which disappeared in 96 h exposed NE (lane 3).

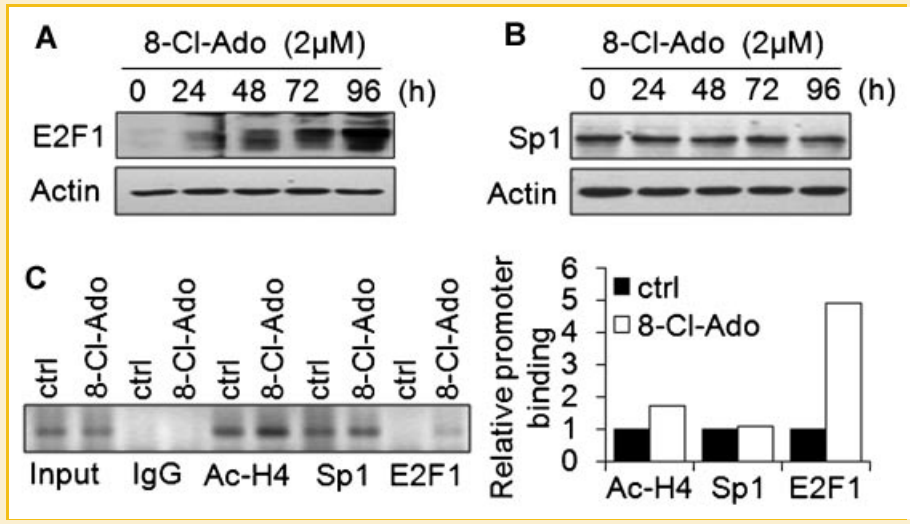

Fig. 2. Induction of E2F1 and its association with activated *p14ARF* promoter in 8-Cl-Ado-exposed H1299. E2F1 (A) and Sp1 (B) proteins were analyzed by Western blotting as described in Figure 1A. Actin as a loading control. C: Increased association of E2F1 with activated *p14ARF* promoter. Cells were unexposed (ctrl) or exposed to 8-Cl-Ado (2 μM) for 96 h. Chromatin was precipitated with antibodies specific to acetyl-histone-H4 (Ac-H4), E2F1, or Sp1. Regular PCR (left panel) and real-time PCR (right panel) were performed using specific primers (P1 and P2) for testing the −281/−56 region (see Fig. 3A). Relative promoter binding in unexposed cells was normalized to 1; n = 3.

We infer that Sp1 might dominantly bind to the overlapping sites before 8-Cl-Ado exposure. To test this hypothesis, we performed competition using the −281/−243 fragment as probe and unexposed NE. Compared with the binding in the absence of competitor (Fig. 4C, lane 2), E2F1 consensus competitor had no significant effects on complexes B–G (lane 3). Obviously, Sp1 consensus inhibited most complexes except complex C (lane 4). These results indicate that the formation of most complexes is attributable to Sp1 binding to the −281/−243 region, except complex C which may be correlated to the binding of unknown

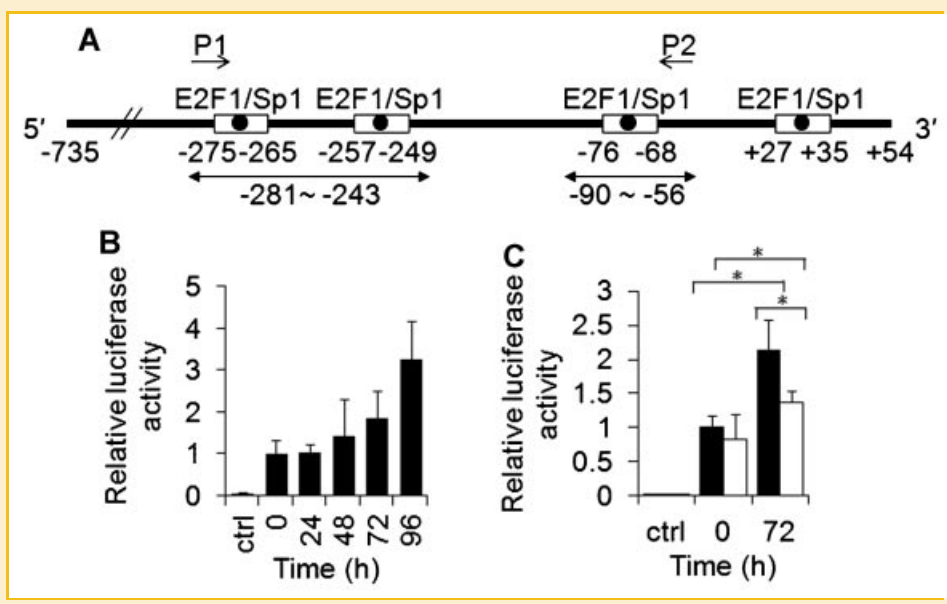

Fig. 3. The role of distal overlapping E2F1/Sp1 site in the *p14ARF* promoter activation. A: Interpretation of the promoter and the relative positions of putative E2F1 (open square) and Sp1 (solid cycle) sites (simple GC boxes not shown). The transcription start site is defined as +1; P1 and P2 indicate the primers for ChIP assay in Figure 2C. B: Activation of the *p14ARF* promoter by 8-Cl-Ado. pGL3ARF(−735/+54) and pRL-CMV plasmids were transfected into H1299. After 24 h transfection, cultures were exposed to 8-Cl-Ado (2 μM). *Firefly* luciferase activities were assayed and verified. The relative *Firefly* luciferase activities in unexposed cells were normalized to 1. C: Identification of the distal overlapping E2F1/Sp1 binding site as a response element. Wild-type pGL3ARF(−735/+54) or mutant pGL3ARF(−735/+54)m was transfected into H1299. After 24 h transfection, cultures were exposed to 8-Cl-Ado for 72 h or left untreated (0 h). The enzyme activities were assayed and normalized. The relative *Firefly* luciferase activities in wild-type pGL3ARF(−735/+54)-transfected/unexposed cells were normalized to 1. Empty-vector-transfected cells were used as a control (ctrl). Data represent mean ± SD (n = 3), $^{*}P < 0.05$.

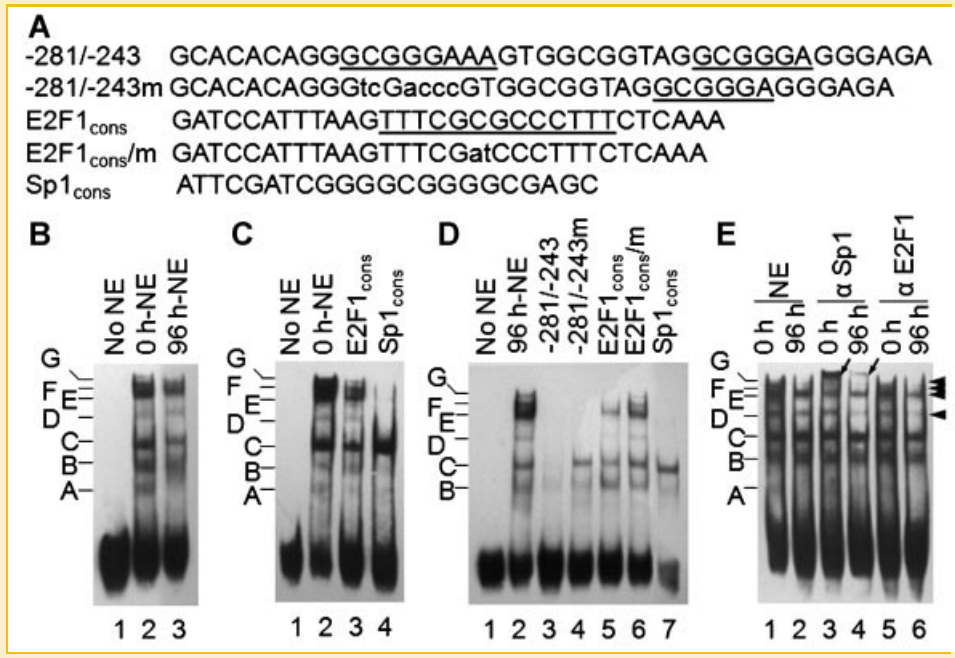

Fig. 4. Formation of multiple Sp1- and E2F1-related complexes on −281/−243 region of the promoter. In EMSA experiments, the probe was biotin-labeled oligonucleotide corresponding to the −281/−243 region of the promoter, which was incubated with 2 μg nuclear extracts (NE), and nucleoprotein–DNA complexes were separated by 6% PAGE. In EMSA supershift analysis, anti-Sp1 or anti-E2F1 antibody was added. A: The oligonucleotides used as probe or competitor in EMSA assays. E2F1-binding site is underlined; lowercase represents mutated nucleotide. B: Formation of multiple protein–DNA complexes in H1299 before (0 h) and after (96 h) 8-Cl-Ado exposure. A–G capitals indicate protein–DNA complexes. C: EMSA competition analysis of protein–DNA interaction before 8-Cl-Ado exposure. Probe was incubated with unexposed (0 h) NE. The competitors (200× M excess) are oligonucleotides containing E2F1-binding consensus (lane 3) and Sp1 consensus (lane 4). D: Competition analysis of protein–DNA interaction after 8-Cl-Ado exposure. The probe was incubated with NE from 96 h exposed H1299. The competitors are indicated above each lane. E: EMSA supershift analyses of Sp1 and E2F1 bindings to the promoter before and after exposure. Sp1–DNA supershift bands are indicated by arrow, and the changed E2F1–DNA complexes are indicated by arrowhead. αSp1, anti-Sp1 antibody; αE2F1, anti-E2F1 antibody; NE, nuclear extracts.

protein(s) to this region. Notably, complex A could be inhibited by both E2F1 consensus and Sp1 consensus. This observation indicates that the complex A is at least in part attributed to Sp1 binding to the −275/−265 overlapping site, although we cannot exclude the contribution of E2F1 binding. Together, these data suggest that Sp1 dominantly binds to overlapping sites before exposure.

Sp1 IS DISPLACED BY E2F1 FROM OVERLAPPING SITE DURING EXPOSURE

Since the induction of E2F1 by 8-Cl-Ado activated the *ARF* gene (Fig. 2), we infer that E2F1 should occupy the overlapping sites in the promoter during 8-Cl-Ado exposure. To test this idea, competition experiments were performed with specific competitors and 96 h exposed NE. Compared with the binding in the absence of competitor (Fig. 4D, lane 2), the B–G complexes were abolished by unlabeled −281/−243 fragment (lane 3). Whereas the −281/−243 mutant, lacking the upstream overlapping site (−275/−265, AGGGCGGGAAA) but keeping a downstream "overlapping site" (−257/−249, taGGCGGGa), inhibited most complexes, but did not affect complex C (lane 4). Interestingly, Sp1 consensus displayed a similar competition (lane 7). These results indicate that the downstream taGGCGGGa motif may function as a GC-box (Sp1 site) does, but yet we cannot differentiate contributions of the bindings of E2F1 and Sp1 to B and D–G complexes. To ascertain E2F1 binding to the overlapping site(s) in −281/−243 region, we performed competition using oligonucleotides containing E2F1 consensus or its mutant. The E2F1 consensus inhibited D, F, and G complexes (lane 5), whereas the mutant could not (lane 6). These results indicate that complexes D, F, and G are attributed to E2F1 binding to the overlapping site after 8-Cl-Ado exposure.

To further confirm the increase of E2F1 binding after exposure and to differentiate its binding from Sp1 bindings, we performed supershift assays with anti-Sp1 and anti-E2F1 antibodies (Fig. 4E). When anti-Sp1 antibody was added into 0 h NE reaction, a thick supershift band on the top of gel was observed, accompanied by disappearance of F and G bands and fading of E band (Fig. 4E, lane 3 vs. lane 1). Compared with 0 h NE reaction (lane 3), 96 h NE reaction gave rise to a very fine supershift band in the presence of anti-Sp1 antibody (lane 4). These results indicate that Sp1 binding is decreased after exposure. Unexpectedly, we did not observe supershift bands in 0 h NE and 96 h NE reactions, when the anti-E2F1 antibody was added into the reactions (lanes 5 and 6). However, it is obvious that the 0 h NE bindings had no significant difference in the presence (lane 5) and absence (lane 1) of anti-E2F1 antibody. Importantly, addition of anti-E2F1 antibody into 96 h NE reaction led to disappearance of F and G bands and reduction of D and E bands, compared with 0 h NE reaction (lane 6 vs. lane 5). The decrease of complexes D–G in 96 h NE reaction by anti-E2F1 antibody suggests that E2F1 binding is increased after 8-Cl-Ado exposure.

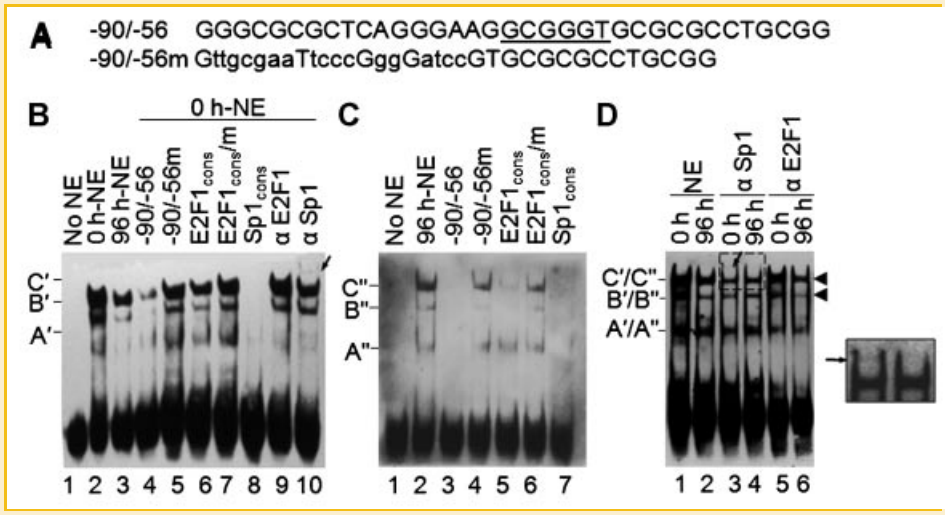

Fig. 5. Increasing binding of E2F1 to the overlapping site after 8-Cl-Ado exposure. A: The probe and its mutant competitor used in EMSA. E2F1 binding site is underlined; lowercase represents mutated nucleotide. B: The −90/−56 fragment containing one overlapping E2F1/Sp1 site sandwiched between two putative Sp1 sites was used as probe, and incubated with unexposed NE. The competitors (200× M excess) are indicated on the top of each lane. αSp1, anti-Sp1 antibody; αE2F1, anti-E2F1 antibody. The arrow on the top of lane 10 indicates supershifted complexes. C: The −90/−56 fragment (probe) was incubated with 96 h exposed NE. The competitors are indicated. D: EMSA supershift analyses of Sp1 and E2F1 bindings to the −90/−56 fragment of the promoter before and after exposure. In supershift assays, anti-Sp1 (αSp1) or anti-E2F1 (αE2F1) antibody was added. Sp1–DNA supershift bands are indicated by arrow, and the changed E2F1–DNA complexes are indicated by arrowhead. The magnified shown indicates the Sp1 supershift band of dash-lined area. Protein–DNA complexes are indicated by capital letters.

To further demonstrate the decrease of Sp1 binding at the overlapping sites after exposure, we performed EMSA using the −90/−56 fragment as a probe, which contains an overlapping E2F1/Sp1 site (−76/−68) (Table I) sandwiched between two Sp1 similarities (Fig. 5A). When the probe was incubated with unexposed NE, three (A′–C′) complexes occurred (Fig. 5B, lane 2). All complexes could be inhibited by unlabeled probe (lane 4) and Sp1 consensus (lane 8), but could not by −90/−56 mutant, E2F1 consensus, and E2F1 consensus mutant (lanes 5–7). The addition of anti-Sp1 antibody led to a supershift complex (lane 10), while anti-E2F1 antibody had no effect (lane 9). These results indicate that Sp1 binding to the −90/−56 region contributes to three complex formation before exposure.

When the same probe was incubated with 96 h exposed NE, three (A″–C″) complexes occurred (Fig. 5C, lane 2). The E2F1 consensus competitor abolished B″ and C″ complexes but not A″ complex (Fig. 5C, lane 5), while E2F1 consensus mutant had no effect on them (lane 6), indicating that B″ and C″ complexes are attributed to E2F1 binding to the overlapping site. Surprisingly, the Sp1 consensus abolished all complexes (lane 7), as unlabeled probe did (lane 3). These results indicate that Sp1 may possibly contribute not only to A″ complex but also to E2F1-associated B″ and C″ complexes. To clarify which one of E2F1 and Sp1 to contribute to these complexes after exposure, we again performed the supershift experiments. The addition of anti-Sp1 antibody into 0 h NE reaction led to a supershift band and reduction of three complexes (Fig. 5D, lane 3 vs. lane 1), indicating Sp1 contribution to the formation of these complexes before exposure. However, addition of the antibody in 96 h NE reaction only reduced C″ complex (lane 4 vs. lane 2) without supershift band occurring (lane 4 vs. lane 3), again indicating decrease of Sp1 binding after exposure. Importantly, addition of anti-E2F1 antibody had no significant effect on these complexes in 0 h NE reaction (lane 5 vs. lane 1), but markedly reduced B″ and C″ complexes in 96 h NE reaction (lane 6 vs. lane 2 and lane 5), indicating E2F1 binding contributes to B″ and C″ complexes. Together, these data indicate that E2F1 binding to the overlapping site is increased but Sp1 binding is decreased after exposure. These data also indicate that E2F1 binding to the overlapping site may require Sp1 occupying the Sp1 sites around the overlapping site.

Sp1 AND E2F1 BIND TO THE OVERLAPPING SITE COMPETITIVELY

To understand better how upregulated E2F1 competes with Sp1 for the overlapping sites, an oligonucleotide, containing a perfect E2F1 consensus motif (TTTCGCGCCCTTT) [Helin et al., 1992] which overlaps with an Sp1 site, was used as a probe and incubated with pure GST-E2F1 fusion protein and recombinant Sp1 in different molar ratios (Fig. 6). Compared with E2F1 binding in the absence of Sp1 (lane 2), the binding of E2F1 to the probe was increased, when E2F1 mole was higher than Sp1 (lanes 3 and 4). When Sp1 mole is higher than E2F1, Sp1 binding was increased, whereas E2F1 binding was inhibited (lanes 6 and 7). To differentiate E2F1 binding from Sp1 binding, we performed the binding of Sp1 to the probe in the absence of E2F1, showing a similar pattern of binding (lane 8) to that when E2F1/Sp1 ratio was 1:4 (lane 7), indicating that the CgCGCCC motif in the E2F1 consensus can be recognized by Sp1. These results suggest that E2F1 and Sp1 compete with one another for the overlapping site and that appropriate Sp1 can increase E2F1 binding.

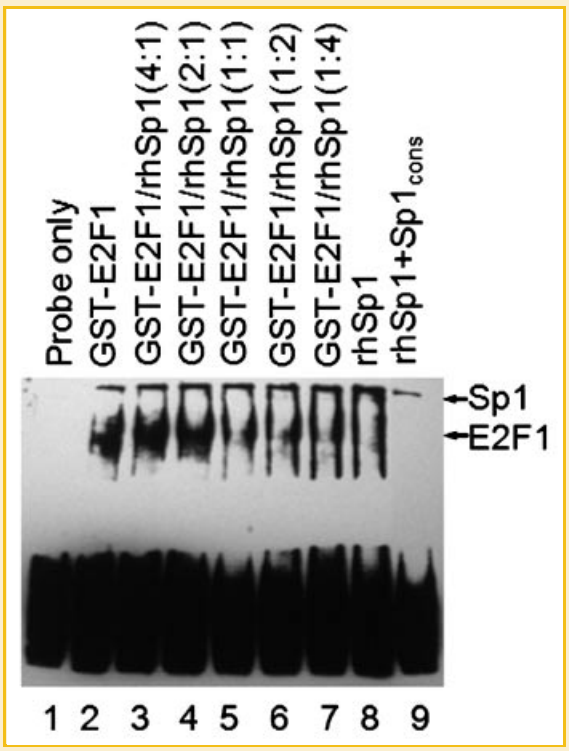

Fig. 6. E2F1 and Sp1 compete with one another for the E2F1/Sp1 overlapping site. A 31-bp synthesized oligonucleotide containing a perfect E2F1 consensus (TTTCgCGCCCTTT) was used as probe, and incubated with varied moles of pure GST-E2F1 fusion protein and/or recombinant human Sp1 (rhSp1) in binding reactions. A maximum of 5 pmol GST-E2F1 (lane 2) or 5 pmol rhSp1 (lane 8) or different mole ratios between E2F1 and Sp1 as indicated in brackets were used (lanes 3–7). To identify the binding of Sp1, competition was performed using oligonucleotides containing Sp1 consensus (lane 9) as a competitor. The arrow indicates Sp1 or E2F1 binding.

DISCUSSION

We demonstrate that the multiple overlapping E2F1/Sp1 sites in the *p14ARF* promoter play crucial role in the cooperative regulation of the *p14ARF* gene by E2F1 and Sp1. 8-Cl-Ado exposure promotes the *p14ARF* gene activation in H1299 cells, which is accompanied by increased expression of E2F1 and constitutive expression of Sp1. Constitutive binding of Sp1 to the overlapping E2F1/Sp1 sites contributes to a constitutive expression of the *ARF* gene at a low level in unexposed H1299, whereas displacing Sp1 from the overlapping sites by E2F1 enhances the gene expression after exposure. These data suggest that E2F1 and Sp1 cooperate to regulate the *p14ARF* gene activation by competition mechanism, in which the overlapping E2F1/Sp1 binding sites play an important role.

The *ARF* gene can be induced by oncoproteins [Sherr, 2001]. The mechanism by which at least some oncogenic signals stimulate *ARF* is thought to be through the activation of E2F-dependent transcription [DeGregori et al., 1997; Bates et al., 1998]. It is widely accepted that the regulation of the *ARF* gene by E2F differs from that of classical E2F target genes required for cell-cycle progression [Dyson, 1998; Komori et al., 2005; del Arroyo et al., 2007]. The capability of the *p14ARF* promoter to distinguish the physiological from the aberrant or supra-physiological levels of E2F1 has recently been attributed to a variant E2F-response element [Komori et al., 2005]. However, a search for *cis*-acting elements cannot find critical E2F binding sites, suggesting that multiple-response elements are present in the promoter [del Arroyo et al., 2007]. In consistent with this, the *p14ARF* promoter contains four putative E2F1 sites at positions −275/−265, −257/−249, −76/−68, and +27/+35 (Table I), and seven putative Sp1 binding sites [Robertson and Jones, 1998]. The competition experiments demonstrated that except to the taGGCGGGa motif at position −257/−249, which is not able to be recognized by E2F1 but the GGCGGG hexamer in the taGGCGGGa motif is an actual Sp1 site (Fig. 4D), at least two E2F1 sites at the positions −275/−265 (AGGGCGGGAAA) and −76/−68 (AAGGCGGGT) could be recognized by both E2F1 and Sp1 factors (Figs. 4 and 5), suggesting they are overlapping E2F1/Sp1 sites. Furthermore, mutation of the −275/−265 overlapping site partially reduced the responsibility of the core promoter to 8-Cl-Ado stimulation (Fig. 3B,C), suggesting that the −275/−265 overlapping site is necessary but not sufficient for the full activity of the core promoter. Alternatively, downstream overlapping sites as well as simple Sp1 sites are required for the full promoter activity.

The *p14ARF* promoter is highly responsive to E2F1 expression [Robertson and Jones, 1998; Parisi et al., 2002; del Arroyo et al., 2007], for which Sp1-like factors are indispensable [Parisi et al., 2002]. The competitive displacement mechanism can explain how E2F1 and Sp1 cooperate to regulate the *p14ARF* gene expression. Decision of which factor to bind to the overlapping site depends on the expression status of competitive partners. In unexposed H1299, both Sp1 and E2F1 were constitutively expressed at basal levels (Fig. 2A, B), but Sp1 was relatively abundant and dominantly bound to the overlapping sites as well as the simple GC-boxes (Figs. 4 and 5). In exposed cells, however, upregulated E2F1 increasingly bound to the overlapping sites (Fig. 5C, D) despite constitutive Sp1 in relative abundance. Sp1 binding to the overlapping E2F1/Sp1 sites abrogates E2F1-dependent *ARF* activation, maintaining a constitutively low expression in H1299 cells, whereas displacing Sp1 at the overlapping sites by increased E2F1 promotes *ARF* activation in response to 8-Cl-Ado. This suggestion is also supported by the ChIP assays (Fig. 2C), in which we showed increased association of E2F1 with the active promoter, although there was no overall change of Sp1 binding to the promoter. Probably, the failure to test the changes at chromatin level could be that Sp1 as a universal factor is constitutively abundant in the nucleus, so that the interaction between antibody and free Sp1 might interfere with chromatin precipitation. It is also possible that there are too much GC-boxes (Sp1 sites) [Robertson and Jones, 1998] but only two or three E2F1/Sp1 sites in the *p14ARF* promoter. Even during the activation of the promoter, Sp1 still binds to multiple simple Sp1 sites (Figs. 4E and 5C). Therefore, it is difficult to differentiate the small change by ChIP method, although the factor departed from the overlapping E2F1/Sp1 sites after drug treatment. Fortunately, we found the decrease of Sp1 binding and increase of E2F1 binding at the overlapping sites after drug treatment in supershift experiments (Figs. 4E and 5D). Undoubtedly, the in vivo displacement of Sp1 at the overlapping sites by E2F1 requires a threshold E2F1 to overcome

Sp1 occupation of the sites. E2F1 and Sp1 competition for multiple overlapping E2F1/Sp1 sites can explain and support the notion [del Arroyo et al., 2007] that in the *ARF* promoter, E2F1 binds to multiple low-affinity response elements that act in concert, in which E2F1 might act in conjunction with other factors such as Sp1.

Sp1-mediated constitutive expression has recently been identified in the human hyaluronan synthase 2 gene [Monslow et al., 2006]. Differently, in the regulation of the *p14ARF* gene, Sp1 is dual-functional regulator, depending on its binding to overlapping sites and/or Sp1 sites. In 8-Cl-Ado-unexposed H1299, Sp1 binds to both sites, mediating constitutive expression; in exposed cells it binds to the simple Sp1 sites, tethering E2F1 binding to the overlapping sites. Since the complex C is not characterized, we do not exclude the possibility that other protein(s) might participate in *ARF* gene regulation.

Usually, the bindings of competitive partners to overlapping site are mutually exclusive. Interestingly, in a binding assay using a probe containing a perfect E2F1 consensus (TTTCgCGCCCTTT) [Helin et al., 1992] overlapping with a Sp1 site, and pure E2F1 and Sp1 proteins in different molar ratios, we found that except exclusion, appropriate Sp1 could increase E2F1 binding to the overlapping site (Fig. 6), suggesting that competition for overlapping site is not simply exclusive, but is fraught with coordination. Sp1 and E2F1 can bind to each other in vitro and in vivo [Karlseder et al., 1996]. It is possible that the Sp1–E2F1 interaction may change E2F1 conformation favorable to binding. Another possibility is that Sp1 leads E2F1 to the recognition sites. In addition, E2F1 phosphorylation can affect the interaction of E2F1 with associated proteins and the ability of E2F1 binding to DNA [Krek et al., 1994], we do not exclude the effect of E2F1 phosphorylation on E2F1-binding ability.

The E2F1, as a transcription factor, functions as heterodimers that bind a consensus sequence TTTSSCGC (S = G or C) [Dyson, 1998] to modulate target gene expression. The Sp1, binding to GC-rich sequences, can activate transcription through a variety of mechanisms, functioning as a basal promoter element and as an upstream activator, depending on promoter context [Fry and Farnham, 1999]. Sp1 and E2F1 cooperate to activate the *TK* promoter by direct interaction, for which a precise spatial arrangement of factors is required [Karlseder et al., 1996]. Similarly, numerous GC-boxes are continuously arranged and the overlapping E2F1/Sp1 sites are sandwiched between them in the *p14ARF* promoter, which are advantageous to the consolidation of E2F1 and Sp1 interaction and cooperation. This can explain why abrogation of Sp1 binding by Sp1 consensus led to inhibition of E2F1 binding to the promoter (Figs. 4D and 5C).

Obviously, the overlapping E2F1/Sp1 site, being present in multiple copies in the *p14ARF* promoter, can serve as the targets for both E2F1 and Sp1, thereby playing a crucial role in response to some oncogenic signals and stimulators, which activate the *ARF* gene through inducing E2F in the cell. Nevertheless, we are far from understanding of the *p14ARF* gene regulation. At present, we have not clarified whether Sp1 and/or E2F1 interact with possible functional factor(s) on the overlapping sites, and we have not defined the upstream sequence of the promoter which is important for the gene activation [Robertson and Jones, 1998].

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