

E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis

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The E2F family of transcription factors plays a pivotal role in the regulation of cell proliferation in higher eukaryotes. We used DNA microarrays and cell lines containing either inducible E2F-1 or inducible E2F-3 to identify novel E2F target genes. Our data indicate that E2F up-regulates the expression of genes not previously described as E2F target genes. A number of these E2F-regulated genes are involved in DNA replication, DNA repair and mitosis. These results suggest that E2F affects cell cycle progression both at S phase and during mitosis. Furthermore, our findings indicate that E2F-dependent gene activation may contribute to the cellular response to DNA damage.

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Keywords: E2F; cell cycle; mitosis; DNA replication; DNA repair; DNA microarrays

Introduction

E2F transcription factors play a crucial role in the control of cell cycle progression and they regulate the expression of genes required for G1/S transition including genes encoding DNA replication proteins, enzymes involved in nucleotide synthesis and components of the origin recognition complex (Dyson, 1998; Nevins, 1998). E2F activity is modulated by multiple mechanisms including negative regulation by interaction with the product of the Rb tumor suppressor gene, RB, and its related proteins p107 and p130 (reviewed in Dyson (1998)).

Binding of RB family members to E2F results in active transcriptional repression of E2F regulated genes and growth suppression. Phosphorylation of RB in late G1 by cyclin dependent kinases leads to dissociation of the RB/E2F complex (Sherr, 1996; Weinberg, 1995). The combination of cessation of repression of some E2F-regulated genes by the RB/E2F complex and the activation of others by the activated E2F constitutes a

major step in promoting G1 exit. The importance of the RB/E2F pathway in controlling cell growth is further emphasized by the mutations often found in components of the RB pathway: over-expression of either cyclin D or Cdk4 or inactivation of either Rb or its upstream regulator p16 are frequently detected in human tumors (Hall and Peters, 1996; Sherr, 1996; Weinberg, 1995).

The E2F transcription factor is a heterodimeric complex consisting of an E2F molecule and its dimerization partner DP. To date, six E2Fs and two DPs have been found in mammalian cells (Dyson, 1998). The biological properties of the E2F/DP heterodimer are believed to be determined by the E2F component and E2F-1, -2 and -3 represent a subgroup of the E2F family. These E2Fs are specifically regulated by RB and not by the RB related proteins, p107 and p130. Their release from RB precedes the activation of E2F responsive genes as well as S phase entry (Moberg *et al.*, 1996), and their overexpression induces quiescent cells to enter S phase (DeGregori *et al.*, 1997; Johnson *et al.*, 1993; Lukas *et al.*, 1996; Qin *et al.*, 1994; Vigo *et al.*, 1999).

It is clear that E2Fs exert their activity primarily via regulation of target gene expression. However, most of the work done so far was focused on a relatively small set of such target genes, implicated mainly in G1/S transition. In order to gain further insight into the biological roles of E2F family members, we used DNA microarray analysis and cell lines containing inducible E2Fs. We show that E2F-1 and E2F-3 activity up-regulates the expression of genes that can be grouped in three functional groups: genes involved in DNA replication, DNA repair and mitosis.

Results

To identify genes whose expression is altered as a result of E2Fs activity we generated cell lines containing an inducible E2F-3. Rat-1a cells were transfected with an expression vector containing a neomycin resistance gene and the E2F-3 coding region cloned downstream to a mutated mouse metallothioneine promoter (Sompayrac and Danna, 1994). Several neomycin resistant clones were isolated in which E2F-3 expression was induced upon Zinc addition and undetectable in its absence, as deter-

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Table 1 Genes regulated by E2Fs

Accession	Gene description	E2F-1						E2F-3					
		Fold change			Difference call			Fold change			Difference call		
		12(I)	12(II)	16(II)	12(I)	12(II)	16(II)	12(I)	12(II)	14(II)	12(I)	12(II)	14(II)
1 AA799328	EST	3.9	2.9	2.4	I	I	I	4.6	3.2	6.6	I	I	I
2 AA799481	EST, embryonic ectoderm development protein (eed)	2.2	2.7	1.9	I	I	I	1.5	1.7	1.8	NC	I	NC
3 AA799779	EST, glyceronephosphate O-acyltransferase (Gnpat)	1.8	4.2	6.3	NC	NC	I	5.1	7.1	3.9	I	MI	MI
4 AA800199	EST	-1.2	-1.2	-1.3	NC	NC	NC	3	-1	5.5	I	NC	I
5 AA800265	EST, breast and ovarian cancer susceptibility protein (Brcal)	10	6.4	6.2	I	I	I	6.3	8.5	4.8	I	I	I
6 AA800456	EST	-1.3	1.1	-1.5	NC	NC	NC	3.5	1.2	4	I	NC	MI
7 AA800878	EST	9.6	15	8.6	I	I	I	3.3	5.9	3.3	NC	NC	NC
8 AA818226	EST, cytochrome c oxidase, subunit IV (Cox4)	2	1.6	3.1	I	NC	I	-1	1.1	-1.2	NC	NC	NC
9 AA819500	EST, replication factor C 4 (RFC4)	2.7	3.4	2.8	I	I	I	2.2	6	5.5	I	I	I
10 AA819793	EST, flag structure-specific endonuclease (Fen-1)	2.3	2.6	2.2	I	I	I	3.4	7	3.9	I	I	I
11 AA848421	EST, uracil-DNA glycosylase (Ung)	2.3	3.3	-4.1	NC	NC	NC	7.1	18.3	4.9	MI	I	NC
12 AA851223	EST, Muscle specific enolase (beta beta enolase) (Eno3)	3.1	3.8	8.2	MI	NC	I	-1	4.7	2.4	NC	I	NC
13 AA858636	EST, MCM2	2.1	2.2	2.1	I	I	I	4	4.6	4.4	I	I	I
14 AA859768	EST	2.5	4.4	2.8	I	I	I	35	13	10	I	I	I
15 AA859898	EST	2.6	3.3	3.8	I	I	I	2.1	2.7	2.9	NC	I	I
16 AA859933	EST	2.5	4.1	3	I	I	I	1.9	3.6	4.3	NC	I	I
17 AA860055	EST	2.6	3	3.4	I	I	I	5	5.1	6.4	I	I	I
18 AA874919	EST, mismatch repair protein, MSH2	10.6	18.7	1.9	I	I	MI	6.2	16.9	4.3	I	I	MI
X93591	Mismatch repair protien, MSH2	2	2.3	1.3	I	I	NC	2.7	3	3.1	I	I	I
19 AA891140	EST	3.1	13.5	13.8	I	I	I	1.3	5.3	2.4	NC	MI	NC
20 AA892248	EST	2.3	2.3	1.6	I	I	NC	-1.6	1.3	-1.4	NC	NC	NC
21 AA892635	EST, GTP-binding protein tc10	5	5.8	5.5	I	I	I	1.4	12.1	2.1	NC	MI	NC
22 AA893590	EST, Ariadne 2 (Ari2)	6.7	1.8	2.1	I	NC	I	-3.6	-1.4	-2.4	NC	NC	NC
23 AA894059	EST, serine/threonine kinase (sak-a)	4.3	3.5	3.1	I	I	I	4.5	2.4	2.1	I	I	NC
24 AA894101	EST, N-terminal Asn amidase (Ntan 1)	-1	1.1	1	NC	NC	NC	4.5	1.1	4	I	NC	I
25 AA894200	EST, non-muscle myosin alkali light chain	2.1	2.4	2	I	I	NC	-1.2	1	-1.1	NC	NC	NC
26 AA899854	EST, topoisomerase II alpha Z19552	2.7	1.9	2	I	I	I	2.2	2.2	3.6	I	I	I
27 AA900769	EST, vaskular alpha-actin X06801	3.4	3.1	2.9	I	I	I	1.8	2.8	4.5	NC	NC	I
28 AA957917	EST, cationic amino acid transporter-1 (CAT-1)	2.5	3.4	3.2	I	I	I	2	2.5	1.8	I	I	I
29 AA996401	EST, high mobility group protein 2 (Hmg2)	4.1	16.4	16.8	I	I	I	4.2	1.5	2.1	NC	NC	NC
A1008836	EST, high mobility group protein 2 (Hmg2)	5.9	5.6	9	I	I	I	1.2	3.4	2.5	NC	NC	NC
D84418	High mobility group protein 2 (Hmg2)	2.9	2.3	2.1	I	I	I	4.6	3.1	4.7	I	I	I
30 AB012214	DNA cytosine 5 methyltransferase rTIM	2.9	1.7	1.9	I	MI	I	4	2.7	5.9	I	I	I
31 AB019576	Nerve growth factor-induced factor A (NGFI-A)	1.9	2.1	2.2	I	I	I	4.1	3.5	4.2	I	I	I
32 AF023087	Nerve growth factor-induced factor A (NGFI-A)	2.4	2.4	2.2	I	I	I	3.2	4.3	3.5	I	I	I
M18416	Nerve growth factor-induced factor A (NGFI-A)	2	2.5	1.5	I	MI	NC	2.9	10.9	5	NC	I	I
33 AF027954	Bcl-2-related ovarian killer protein (Bok)	2.2	2.8	3.1	I	I	I	1.2	-1	-1.2	NC	NC	NC
34 AF035951	Kinesin-related protein KRP1 (KRP1)	4.2	3.6	5.8	I	MI	I	1.9	-1	1.1	NC	NC	NC
35 AI044390	EST, nuclear localization signal binding protein (NBP60)	2	2.8	2.3	MI	MI	I	4	3.7	1.8	NC	MI	NC
AI145490	EST, nuclear localization signal binding protein (NBP60)	2.8	3.1	4.4	I	I	I	16.5	18	10.6	I	I	I
36 AI071299	EST, zinc finger transcription factor homolog CPG20	1.3	1.6	1.4	NC	I	NC	3.3	3.4	-1.1	I	I	NC
		2.8	2.6	2.1	I	I	I	2.7	-1.4	2.6	NC	NC	NC
		1.1	1.2	1.2	NC	NC	NC	5.7	-1.6	3.8	I	NC	I

Continued

Table 1 (Continued)

Accession	Gene description	E2F-1						E2F-3					
		Fold change			Difference call			Fold change			Difference call		
		12(I)	12(II)	16(II)	12(I)	12(II)	16(II)	12(I)	12(II)	14(II)	12(I)	12(II)	14(II)
37 AI102562	EST	1.4	1.9	1.1	NC	NC	NC	3.4	2.7	3.9	I	I	I
38 AI171243	EST, p14-subunit of replication protein A (RPA3)	2.6	1.8	1.6	I	I	NC	3.1	5.4	8.6	I	I	I
39 AI171734	EST, mitochondrial fumarase J04473	10.5	7.1	4.9	I	I	I	3.8	2.5	3.7	NC	NC	NC
40 AI639042	Mitochondrial fumarase	2.1	2	1.9	I	I	I	1.1	1.6	1.3	NC	NC	NC
41 AI639082	EST, MCM6	2.2	2.1	2.2	I	I	I	2.2	2.9	2.7	I	I	I
U17565	EST, MCM6	1.7	1.7	1.7	I	I	I	4.9	5.5	5.7	I	I	I
U17565	EST, MCM6	2	1.6	1.4	I	I	NC	5.4	6	5.6	I	I	I
42 AI639142	EST, MCM6	2.1	2	2	I	I	I	5.9	8.3	7.8	I	I	I
43 AI639172	EST, stem-loop binding protein (Slbp)	3.3	4.9	3.9	I	I	I	3.8	3.4	5.5	I	I	I
44 AJ011606	DNA polymerase alpha subunit II	2	3.3	2.7	I	I	I	3	3.1	3.2	I	I	I
45 AJ011607	DNA polymerase alpha subunit III (primase)	2.8	3.2	2.6	I	I	I	3.9	6.3	3.9	NC	I	I
AJ011607	DNA polymerase alpha subunit III (primase)	2	2.6	1.8	I	I	I	2.5	2.6	2.2	I	I	I
46 AJ011608	DNA polymerase alpha subunit IV (primase)	19.4	3.7	3.4	I	I	I	6.4	13.9	5.9	NC	I	I
U67994	DNA primase small subunit	2.1	2.1	1.8	I	I	I	2.8	2.9	2.6	I	I	I
47 AJ223355	Mitochondrial dicarboxylate carrier	3	3.6	4.2	I	I	I	2.8	4.1	3.7	I	I	I
48 D13417	Transcription factor HES-1	2.3	2.6	7.1	I	I	I	2.9	2.5	2.4	MI	NC	NC
49 D14015	Cyclin E	1.5	1.4	1.4	NC	NC	NC	7.4	9.5	2.2	I	MI	NC
D14015	Cyclin E	2.8	2.7	2	I	I	I	2.2	2.3	2	MI	I	NC
50 D31873	LIMK-1	4.6	4.9	3.2	I	I	I	3.8	4.1	3.6	I	I	I
51 D89731	AIM-1	12.8	9.1	11.1	I	MI	NC	-1.4	5.3	2.2	NC	NC	NC
52 H31604	EST, mitotic checkpoint protein kinase BUB1B (Bub1b)	2	2.7	2.7	I	I	I	1.9	1.8	3.3	NC	NC	I
53 H31955	EST	2.5	3.2	3.3	I	I	I	3.9	8.1	5.8	I	I	I
54 K01932	Glutathione S-transferase Yc1 subunit	2.1	3.5	2.7	MI	I	I	1.2	2	1.6	NC	NC	NC
S72505	Glutathione S-transferase Yc1 subunit	23.3	16.8	13.3	I	I	I	1.8	1.7	2.4	I	I	I
X78848	Glutathione S-transferase Yc1 subunit	17.6	11.7	9.4	I	I	I	1.7	2.2	2.1	NC	I	MI
55 L12138	Thymidylate synthase	5.9	23.5	12.9	I	I	I	2.1	2.4	2.4	I	I	I
56 L22294	Pyruvate dehydrogenase kinase	2.3	3.2	3.3	I	NC	I	5.6	5.8	3.8	MI	MI	I
57 L31840	NUP107	3	2.9	2.6	I	I	I	2.1	3.1	5	MI	I	I
58 M13979	Brain glucose-transporter protein	3.2	6.1	5	I	I	I	3.5	3.2	7.8	MI	I	I
59 M24604	Proliferating cell nuclear antigen (PCNA)	2	2.6	2.6	I	MI	I	1.1	1.6	1.6	NC	NC	NC
60 M58040	Transferrin receptor	2.5	2.2	1.9	I	I	I	4.8	6	6	I	I	I
61 M83107	SM22	8.1	11.9	6.4	I	I	NC	2.7	7	3.2	I	I	I
M83107	SM22	3.3	6.5	5	I	I	I	7.3	20.1	6.9	I	I	I
62 M98327	Transfer RNA-Valine synthetase	27	41.9	44	I	I	I	12	38	11.7	I	I	I
63 U18314	Lamina associated polypeptide 2 (LAP2)	4.1	1.9	2.4	I	NC	MI	1.8	2.1	3.3	NC	NC	I
U18314	Lamina associated polypeptide 2 (LAP2)	2.3	3.3	2.9	I	I	I	3	4.1	4.2	I	I	I
64 U38379	Gamma-glutamyl hydrolase precursor	3.8	3.6	3.2	MI	MI	I	4.3	7.4	2.3	MI	I	MI
65 U44979	Kinesin-related protein 2 (KRP2)	3.5	5.1	3.3	I	I	I	2.4	2.5	3.2	MI	I	I
66 U45986	Myx	3.4	5.6	9.9	I	NC	I	1.3	1.7	2.2	NC	NC	NC
67 U64030	dUTPase	4.2	14.2	18.1	I	MI	I	3.5	1	-1.4	NC	NC	NC
68 U72353	Lamin B1	1.8	1.3	1.5	I	I	I	3.4	5.2	7.8	I	I	I
69 U75920	APC binding protein EB1	1.8	2.7	2.6	I	I	I	5.4	2.8	3.7	I	I	I
70 X60767	Cdc2A	1.2	1.7	1.3	NC	I	NC	5.1	1.9	4.9	I	NC	I
71 X98490	p32-subunit of replication protein A (RPA2)	1.9	2	2.2	I	I	I	3	3.9	5.1	I	I	I
72 Z21780	nup155	2.6	3	2.2	I	I	I	4.7	4.1	5.3	I	I	I
		2.7	3.7	2.4	I	I	I	6.7	2.5	3.8	I	I	I
<i>Decreased genes</i>													
1 AA800062	EST, acid ceramidase (LOC56352)	-2	-1.8	-2.7	D	NC	MD	-1.4	-1.4	-1.8	NC	NC	NC
2 AA800576	EST	-2	-2.3	-1.9	D	D	D	-1.4	-1.9	-1.9	NC	NC	D
3 AA800795	EST	-2.5	-8.8	-5.4	D	D	NC	-2.4	-2.7	-2.1	NC	NC	NC

Continued

Table 1 (Continued)

Accession	Gene description	E2F-1						E2F-3					
		Fold change			Difference call			Fold change			Difference call		
		12(I)	12(II)	16(II)	12(I)	12(II)	16(II)	12(I)	12(II)	14(II)	12(I)	12(II)	14(II)
4 AA858617	EST, TC10-like Rho GTPase	-3.6	-2.7	-3.4	D	D	MD	-1.8	-1.9	-1.8	NC	NC	NC
5 AA866299	EST	-2	-1.4	-2.1	D	NC	D	1.1	-1.1	1.2	NC	NC	NC
6 AA875033	EST, fibulin 5 (Fbln5)	-2.2	-2	-1.8	D	D	D	-1.8	-2.6	-2.7	D	D	D
7 AA891834	EST	-2.2	-1.6	-2	D	NC	D	-1.1	-1.5	-1.6	NC	NC	NC
8 AA892280	EST	-2.4	-2	-1.3	D	D	NC	-1.5	-2.8	-1.9	NC	D	D
9 AA892378	EST	-2.2	-1.9	-2	D	NC	D	-1	-1.6	1.1	D	D	NC
10 AA892921	EST	-2.2	-2.2	-1.9	D	D	D	-1.7	-2.6	-1.2	D	D	NC
11 AA893022	EST	-2.1	-3.4	-1.9	D	D	NC	-1.9	-1.7	-1.8	NC	NC	MD
12 AA893230	EST	-2.3	-3.6	-3.2	D	D	D	0	-5.8	0	NC	D	NC
13 AA893869	EST	-2.1	-6	-2.7	D	MD	NC	-3.4	-1.2	-1.9	NC	NC	NC
14 AA946439	EST, Histone H4	-2.2	-2.4	-2.3	D	MD	D	-2.3	-1.6	-1.6	D	NC	NC
15 AB009999	CDP-diacylglycerol synthase	-3.6	-2.1	-2.3	D	MD	D	-1.6	-1.5	-1.3	D	NC	NC
16 AB016532	rPER2	-8.4	-9.2	-6.1	D	D	NC	-1.7	-1.3	-1.6	NC	NC	NC
17 AF002251	Maxp1	-2	-6.1	-2	D	MD	MD	-1.6	-2.2	-1.8	D	D	D
18 AF053312	CC chemokine ST38 precursor	-2.3	-1.4	-2	D	NC	D	-3	-2.7	-3.2	D	D	D
19 AI009098	EST, oxygen regulated protein (ORP150)	-1.4	-3	-1.6	NC	NC	NC	-2.6	-3.4	-2.4	D	D	D
20 AJ005396	Collagen alpha 1 type XI	-4.2	-6.5	-5.2	D	D	D	-1.9	-2.2	-2.4	NC	D	D
21 D13978	Argininosuccinate lyase	-2	-4.5	-2.7	D	MD	NC	-3.9	-6.5	-3.7	NC	D	NC
22 E12275	Oxidosqualene lanosterol-cyclase	-3.9	-20.7	-1	D	MD	NC	-13	-7.2	-2.9	D	NC	NC
23 J03179	D-binding protein	-5	-13.8	-2	D	D	NC	-1.4	-2.4	-1.6	NC	MD	NC
J03179	D-binding protein	-4.1	-24.4	-1.6	MD	D	NC	-1.5	-1.7	-1.7	NC	NC	NC
24 J05499	L-glutamine amidohydrolase	-2	-2.5	-2.5	D	D	D	-5.5	-3.5	-2.9	D	D	NC
25 L07315	Dipeptidase (dpep1)	-4.5	-4	-3.4	D	D	D	-5.5	-3.5	-2.9	D	D	NC
L07315	Dipeptidase (dpep1)	-4.5	-4	-3.4	D	D	D	-2	-3.1	-1.6	D	D	NC
26 M21770	Asialoglycoprotein receptor (ASGP)	2.5	-1.1	-5.3	NC	NC	NC	-1.1	-2.2	-1.7	NC	D	NC
27 M35297	G protein coupled receptor	-2	-2.4	-12.2	D	D	MD	-2.2	-2.5	-3.2	D	D	D
28 M38135	Cathepsin H (RCHII)	-2.6	-1.6	-2.1	D	NC	D	-1.5	-2	-2.1	NC	D	D
29 S63521	Glucose-regulated protein GRP78	-2.3	-2.1	-1.4	D	D	NC	-1.6	-1.9	-1.4	NC	D	NC
30 S85184	Cyclic Protein-2 (CP-2)= cathepsin L proenzyme (Ctsl)	-2.1	-2.6	-1.7	D	D	D	-1.7	-1.6	-1.6	D	D	D
31 U03491	Transforming growth factor beta-3	-2.1	-2.8	-1.5	D	D	NC	-7.7	-7.7	-3	NC	NC	NC
U03491	Transforming growth factor beta-3	-2.2	-3.3	-1.6	D	D	NC	-1.8	-2.6	-2.2	D	D	D
32 U23407	Cellular retinoic acid-binding protein II (CRABP II)	-5.4	-2	-8.8	D	D	D	-1.7	-1.2	-4.7	NC	NC	NC
33 U47031	P2x4 ATP receptor	-2.4	-2.4	-2.5	D	D	D	-1.9	-1.7	-1.5	MD	NC	NC
34 U53706	Mevalonate pyrophosphate decarboxylase	-2	-2	-1.4	D	D	NC	-2	-3.1	-2.5	D	D	D
35 U89282	Telomerase protein component 1 (TLP1)	-2.4	-2.9	-2.5	D	MD	NC	-2.3	-4.3	-2.5	D	D	NC
36 X53363	Calreticulin	-2.2	-3.8	-3.2	D	D	D	-2.6	-4.2	-3.3	D	D	D
37 X59737	Ubiquitous mitochondrial creatine kinase	-3.2	-3.1	-2.2	D	D	D	-6.7	-8.5	-3	MD	D	D
38 X82152	Fibromodulin	-2.9	-6.2	-5.8	MD	D	D	-12	-2.4	-7.8	D	NC	MD

mined by Western blot analysis (data not shown and Figure 1). One such clone, Rat-1-MT-HA-E2F-3, was used for further characterization. In addition, we used a Rat-1a derived cell line containing a stably integrated Zinc inducible E2F-1, Rat-1-MT-E2F-1 (Qin *et al.*, 1994). When these cell lines were kept for 48 h in medium with 0.1% serum they both entered quiescence, as determined by FACS analysis (Figure 1 and Kalma *et al.* (2001)). Under these conditions, addition of ZnCl₂ (100 μM) resulted in a rapid and significant elevation of E2Fs protein levels. E2F-3 protein level was increased 4 h after ZnCl₂ addition and it remained high for at least 12 h (Figure 1). E2F-1 protein level was similarly elevated (Kalma *et al.*, 2001). The increase in level of either E2F-1 or

E2F-3 in these quiescent fibroblasts was followed by an S phase entry that was evident 12 h after Zinc addition (Figure 1 and Kalma *et al.* (2001)). Total RNA was extracted from Rat-1-MT-E2F-1 and Rat-1-MT-HA-E2F-3 growth arrested cells before and after E2F induction in four independent experiments. For Rat-1-MT-E2F-1 cells, in one experiment RNA was extracted 12 h after E2F-1 induction and in the other at two time points after E2F-1 induction: 12 and 16 h. For Rat-1-MT-HA-E2F-3, in one experiment RNA was extracted 12 h after E2F-3 induction and in the other 12 and 14 h after E2F-3 induction. In all experiments S phase entry was evident at 12 h (Figure 1 and data not shown). These RNAs were used to prepare probes to screen an Affymetrics Rat DNA

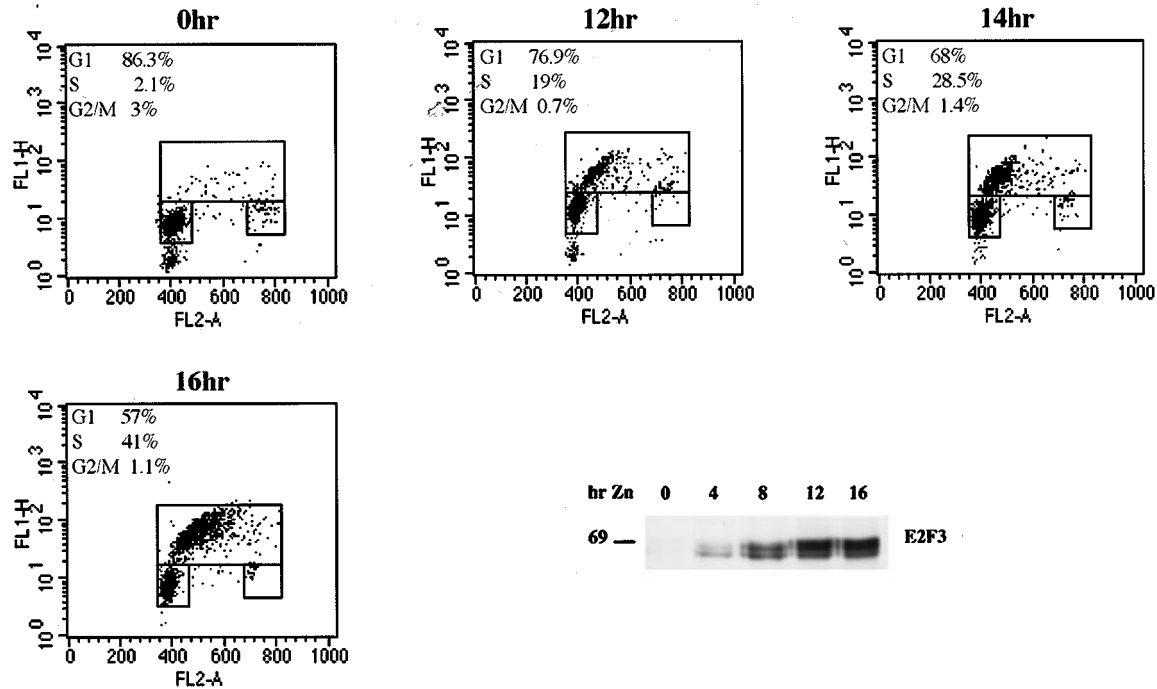


Figure 1 E2F-3 induces cell-cycle progression. Rat-1-MT-HA-E2F-3 cells were kept for 48 h in medium containing 0.1% serum and then 100 μ M ZnCl₂ was added. At the times indicated at the top of each graph (in hours), cells were harvested. Cell cycle distribution was measured by the Cell-Cycle Flow Cytometry Assay (FACS). The graphs depict cell cycle distribution of living cells. The lower right panel depicts E2F3 protein levels. Cells were treated the same as for FACS analysis, lysed and subjected to Western blot analysis with an anti HA monoclonal antibody

microarray (Rat U34A array) containing 8700 cDNAs and expressed sequence tags (ESTs).

Analysis of the data was performed using the Affymetrix GeneChip expression analysis algorithm (version 3.1). Several criteria were used to identify genes whose expression was affected by E2F activity. First, the fold change of each gene, as provided by the Affymetrix analysis, was above a determined threshold both in the experiment that included a single time point after induction and in one of the two time points in the other experiment. The threshold employed in each case was such that yielded a $P > 0.05$ in a Chi-Square test. Thus, the fold change of E2F responsive genes was more than 2 or less than -2 for E2F-1 and more than 3 or less than -3 for E2F-3. The second criteria employed was that the difference call, as provided by the GeneChip software was moderately induced (MI) or induced (I) for up-regulated genes, and, moderately decreased (MD) or decreased (D) for down regulated genes. In addition, only up-regulated genes whose absolute call was 'present' in the experiment (i.e. induced cells) and down-regulated genes whose absolute call was 'present' in the control (i.e. un-induced cells) were considered as E2F responsive genes. These criteria ensured that only genes whose expression was reproducibly altered were considered as E2F responsive genes and the number of false positives was low. The sequences of E2F regulated ESTs were used to scan the non-redundant (nr) database, using the blast

program (NCBI). The threshold E-value used to annotate each EST was 1×10^{-50} .

After employing the above-mentioned criteria we concluded that, out of the 8700 genes and ESTs present on the DNA microarray, expression of 58 genes and 14 ESTs was up-regulated and expression of 28 genes and 10 ESTs was down-regulated in response to the induction of at least one of the two E2Fs studied (i.e. E2F-1 or E2F-3). These genes and ESTs are listed in Table 1. The gene description of annotated ESTs is included.

Out of the 58 up-regulated genes 11 genes were present twice on the DNA microarray and three genes were present three times. These genes were induced in all cases. Out of the 28 down-regulated genes three were present twice on the DNA microarray and their expression was reduced in both cases.

A number of genes were previously identified as E2F target genes (Lavia and Jansen-Durr, 1999; Nevins, 1998) and 16 of them were present on the DNA microarray used for this study. Analysis of their expression pattern revealed that nine of them were up-regulated upon E2F induction. These are DNA polymerase α subunit II, PCNA, Thymidilate synthetase, cdc2, cyclin E, UNG, BRCA1, MCM2 and MCM6 (see Table 1). Expression of another gene, encoding the catalytic subunit of DNA polymerase δ , was up-regulated, yet its absolute call was 'absent' in the experiment and therefore it did not meet our criteria for E2F-regulated genes. Four other known

Table 2 Functional groups of genes up-regulated by E2Fs

Functional group	Gene	Function	Fold change E2F-1	Fold change E2F-3	
DNA repair	UNG	Uracil-DNA glycosylase, BER	2.3/3.3/-4.1	7.1/18.3/4.9	
	MSH2	Mismatch repair	10.6/18.7/1.9	6.2/16.9/4.3	
	MSH6	Mismatch repair	ND/2.7/1.9	ND/ND/2.5	
	PMS2	Mismatch repair	ND/6.3/4.7	ND/ND/3.3	
	BRCA1	Breast cancer susceptibility	10/6.4/6.2	6.3/8.5/4.8	
	Fen-1	Endonuclease, BER	2.3/2.6/2.2	3.4/7/3.9	
	Replication factor C 4 (RFC4)	Replication factor, BER	2.7/3.4/2.8	2.2/6/5.5	
	PCNA	Auxiliary protein of DNA polymerase, BER	2.5/2.2/1.9	4.8/6/6	
	Replication protein A2 (RPA2)	ssDNA binding protein, NER	2.6/3/2.2	4.7/4.1/5.3	
	Replication protein A3 (RPA3)	ssDNA binding protein, NER	2.6/1.8/1.6	3.1/5.4/8.6	
	DNA polymerase delta 2 ^a	Regulatory subunit, BER	ND/3.8/3.7	ND/ND/3.1	
	Mitosis	SAK-a ^a	Serine/threonine kinase	ND/2.8/2.9	ND/ND/4.3
		BUB1b	Mitotic check-point protein kinase	4.3/3.5/3.1	4.5/2.4/2.1
AIM-1		Chromosome segregation, kinase	ND/5.6/4	ND/ND/6.5	
Ki-67		Mitotic antigen	2.5/3.2/3.3	3.9/8.1/5.8	
KRP1		Mitotic motor protein	2/2.7/2.7	1.9/1.8/3.3	
KRP2		Mitotic motor protein	ND/2.3/2.5	ND/ND/5.2	
EB1		APC binding protein	2.8/3.1/4.4	16.5/18/10.6	
Cdc2		Cyclin dependent kinase	3.4/5.6/9.9	1.3/1.7/2.2	
			1.2/1.7/1.3	5.1/1.9/4.9	
		1.9/2/2.2	5.1/3/3.9		

^aThe gene was present twice on the DNA microarrays. ND: not done

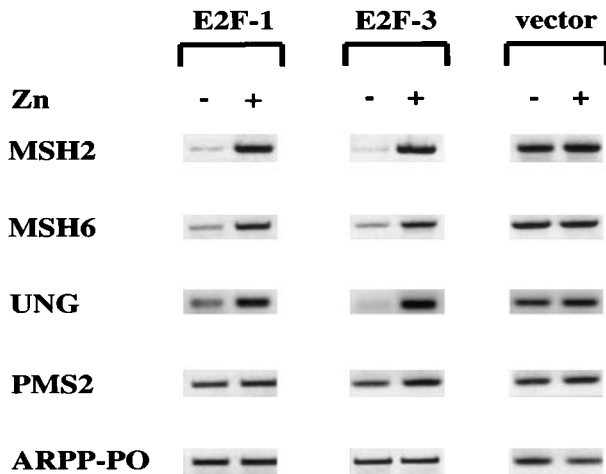


Figure 2 mRNA levels of DNA repair genes are up-regulated by E2F. Rat-1-MT-E2F-1 (E2F-1), Rat-1-MT-HA-E2F-3 (E2F-3) and Rat-1a cells containing an empty vector (vector) were kept in medium with 0.1% FCS for 48 h and then treated with 100 μ M ZnCl₂ for 12 h (+) for E2F-3 and vector, 6 h (+) for E2F-1 or not treated (-) prior to RNA extraction. Total RNA was used for RT-PCR analysis using MSH2, MSH6, UNG, PMS2 and ARPP-PO specific oligonucleotides. ARPP-PO served as an internal control

E2F target genes (DNA polymerase α subunit I, *c-myc*, *N-myc* and p21) exhibited non-specific hybridization. Expression of two additional genes, cyclin D1 and Rb, was not changed. Altogether, these results demonstrate that most of the previously known E2F target genes, that exhibited a specific hybridization, were picked up by our screen and the number of false negative results is low. Fold induction of most of these previously known E2F target genes was 2–3-fold, as the threshold determined by our statistical analysis. This further

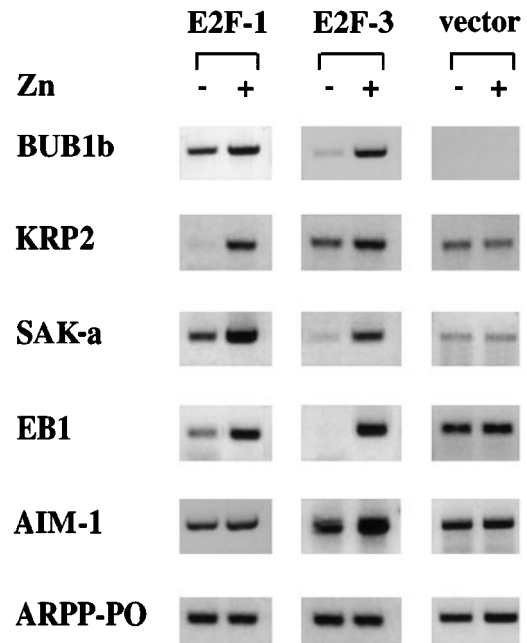


Figure 3 mRNA levels of mitosis related genes are up-regulated by E2F. Rat-1-MT-E2F-1 (E2F-1), Rat-1-MT-HA-E2F-3 (E2F-3) and Rat-1a cells containing an empty vector (vector) were kept in medium with 0.1% FCS for 48 h and then treated with 100 μ M ZnCl₂ for 12 h (+) or not treated (-) prior to RNA extraction. Total RNA was used for RT-PCR analysis using BUB1b, KRP2, SAK-a, EB1, AIM-1, and ARPP-PO specific oligonucleotides. ARPP-PO served as an internal control

supports the notion that such induction of new putative E2F target genes, although low, is most likely meaningful and significant.

Among the other induced genes not previously described as E2F target genes, three functional groups

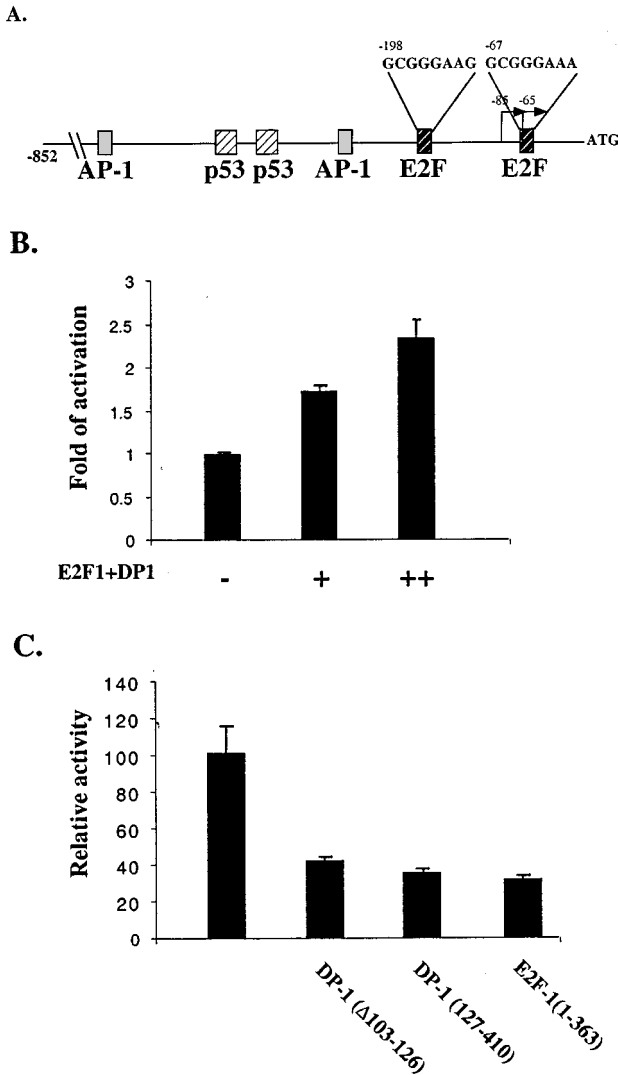


Figure 4 The MSH2 promoter is activated by E2F-1. (a) Schematic representation of putative binding sites of transcription factors in the promoter of human MSH2. The transcription start sites are marked by arrows. Binding sites of AP-1 and p53 as well as putative E2F binding sites are indicated by boxes. (b) H1299 cells were transiently transfected with 0.3 μ g of MSH2-Luc together with 0.5 μ g of pCMV- β -GAL. 0.05 μ g pCMV-DP1 and 0.05 μ g (+) or 0.1 μ g (++) pCMV-E2F-1 were added where indicated. Forty two hours post transfection cell extracts were prepared and used for β -GAL and Luciferase assays. Fold of activation in the luciferase assay, after normalization for β -GAL activity, is depicted in the bar graph. Fold of activation is relative to the sample without exogenous E2F-1/DP-1. The presented data are representative of three independent experiments performed in duplicates. (c) H1299 cells were transiently transfected with 0.5 μ g of MSH2-Luc together with 0.5 μ g of pCMV- β -GAL. One microgram of expression vectors for DP-1(Δ 103-126), DP-1(127-410) or E2F-1(1-363) was added where indicated. Forty two hours post transfection cell extracts were prepared and used for β -GAL and luciferase assays. Luciferase activity after normalization for β -GAL activity is depicted in the bar graph relative to the sample without exogenous E2F-1/DP-1. The presented data are representative of two independent experiments performed in duplicates

of genes were identified. One group included six genes involved in DNA replication, a process known to be regulated by E2F. This group has been described in

our previous study (Kalma *et al.*, 2001). The other two functional groups of putative E2F-regulated genes were genes involved in DNA repair and genes that function during mitosis (Table 2). Among the genes whose expression was reduced upon E2Fs induction no functional groups were identified, and the effect of E2F on the regulation of these genes was not further studied.

Some of the RNAs isolated from both Rat-1-MT-E2F-1 and Rat-1-MT-HA-E2F-3 cells were hybridized, in addition to Rat U34A array, to a second Affymetrics Rat DNA microarray (Rat U34B array) containing 8700 ESTs. These were RNAs from Rat-1-MT-E2F-1 cells 12 and 16 h after induction of E2F-1 and RNA from Rat-1-MT-HA-E2F-3 cells 14 h after E2F-3 induction. Analysis of the data obtained using this microarray, employing similar criteria to those employed for DNA microarray U34A, indicated that expression of 60 ESTs was up-regulated by the E2Fs and expression of two ESTs was down regulated (data not shown).

However, additional experiments are, most probably, required to verify the results obtained using this microarray. Nevertheless, upon annotation of the E2F-regulated ESTs it became apparent that three of the identified genes, PMS2, MSH6 and the gene encoding the regulatory subunit of DNA polymerase δ , were involved in DNA repair and two other identified genes, SAK-a and Ki-67, were mitotic genes. These genes are included in Table 2.

To confirm the array results, RT-PCR was performed using total RNA prepared from growth arrested Rat-1-MT-E2F-1 and Rat-1-MT-HA-E2F-3 cells before and after addition of Zinc (100 μ M ZnCl₂).

Expression of four DNA repair genes and five mitotic genes was studied. The results shown in Figures 2 and 3 for DNA repair genes and mitotic genes, respectively, demonstrate that expression of all the analysed genes was indeed up-regulated upon induction of either E2F-1 or E2F-3. Strong induction was detected for MSH2, MSH6, UNG, BUB1b, KRP2 SAK-a and EB1. Induction of PMS2 by both E2Fs and induction of AIM-1 by E2F-1 was weak but reproducible.

The possible involvement of E2Fs in the regulation of MSH2 expression was further studied by analysing the effect of E2F-1 expression on MSH2 promoter activity. The MSH2 promoter has been isolated and characterized (Iwahashi *et al.*, 1998; Scherer *et al.*, 1996). Our examination of the MSH2 promoter region revealed two putative E2F DNA binding sites at positions -198 and -67. The site at -67 matches perfectly the E2F consensus binding sequence (TTTSSCGC) and the other site differs from the consensus at one position but is identical to E2F sites in known E2F responsive promoters (Figure 4a). An 852 bp fragment spanning the MSH2 promoter from -852 to -1 was synthesized by PCR using human genomic DNA of T98G cells as a template and cloned in pGL2Basic-Luc resulting in MSH2(-852/-1)Luc.

The role of E2F-1 in controlling the MSH2 promoter activity was then assessed by co-transfection of MSH2(-852/-1)Luc and E2F-1 and DP1 expression plasmids, into human lung carcinoma cells, H1299. This co-transfection resulted in a 2.4-fold increase in luciferase activity over the basal promoter activity (Figure 4b), indicating that the MSH2 promoter is an E2F responsive promoter. Furthermore, co-expression of either one of two dominant negative mutants of DP-1 or a dominant negative mutant of E2F-1 together with MSH2(-852/-1)Luc resulted in a 2.4–3.2-fold decrease in promoter activity. The two DP-1 mutants are distinct, but both are defective for DNA binding while retaining the ability to bind E2F and thus they interfere with the activity of all endogenous E2Fs (Wu *et al.*, 1996). The ability of dominant negative DP-1 and E2F-1 mutants to reduce MSH2 promoter activity implies that endogenous E2Fs regulate MSH2 expression. These data strongly suggest that MSH2 is directly regulated by E2F.

Previous analysis of E2F-1 and E2F-3 DNA binding activity along the cell cycle as well as the phenotypes of E2F-1 and E2F-3 deficient mice suggest that E2F-1 and E2F-3 have distinct biological activities (Field *et al.*, 1996; Humbert *et al.*, 2000; Leone *et al.*, 1998; Yamasaki *et al.*, 1996). Such functional differences may arise from their ability to regulate different target genes. However, our study did not identify genes that are regulated exclusively by either E2F-1 or E2F-3. DNA microarray data suggested that some genes were regulated exclusively by a distinct E2F, however, RT-PCR or Northern blot analysis demonstrated that expression of such target genes was, in fact, regulated by both E2Fs (data not shown). Such lack of specificity is in line with two other recent studies identifying genes induced by E2F-1, -2 and -3 (Ishida *et al.*, 2001; Muller *et al.*, 2001).

Discussion

In this study, DNA microarrays and cell lines with inducible E2Fs were employed to identify genes whose expression is regulated by E2Fs. Our analysis demonstrates that out of the 8700 genes and ESTs present on the DNA microarray, expression of 58 genes and 14 ESTs is reproducibly elevated by E2F-1 and/or E2F-3.

Many of the E2F-induced genes identified in this study function in one of three distinct cellular processes: DNA replication, DNA repair and mitosis. Thus, these E2F-induced genes can be grouped in three functional groups. The first functional group, namely genes involved in DNA replication, was described and analysed in our previous study (Kalma *et al.*, 2001). The regulation of DNA replication genes by E2Fs is in agreement with the well documented role E2Fs play in S phase entry, nevertheless, these data provide novel links between E2F and components of the DNA replication machinery.

A second functional group of E2F-regulated genes, identified in this study, includes genes that function

in DNA repair. Some of these genes, such as MSH2, MSH6 and PMS2, function in mismatch repair, a process that repairs mainly replication-related errors. Others, such as replication factor C4 (RFC4), replication protein A subunits 2 and 3, Fen-1 and PCNA, function both in DNA repair and in DNA replication. A possible explanation of the up-regulation of these DNA repair genes is that their function is largely replication-related and they are induced by E2F as part of its effect on DNA synthesis-related genes. However all of the E2F-regulated DNA repair genes included in Table 2, function also in replication-unrelated repair processes such as base excision repair (BER) and nucleotide excision repair (NER) (Norbury and Hickson, 2001; Wood *et al.*, 2001). This raises the possibility that E2F plays a role in the response to DNA damage. This is supported by the E2F-induced up-regulation of UNG and BRCA-1 (Haug *et al.*, 1998; Walsh *et al.*, 1995; Wang *et al.*, 2000 and this study), two DNA repair genes that do not play any known role in DNA replication. Recent reports demonstrating that various DNA damaging agents induce an increase in E2F-1 protein level and DNA binding activity, further support a possible involvement of E2F in the response to DNA damage (Blattner *et al.*, 1999; Hofferer *et al.*, 1999; O'Connor and Lu, 1999).

A third functional group of E2F-induced genes includes eight mitotic genes: (1) SAK-a, a serine/threonine kinase that is a member of the polo family of mitotic regulators and mice deficient of it exhibit a late mitotic failure (Fode *et al.*, 1994; Hudson *et al.*, 2001); (2) BUB1b, a recently identified member of the Bub1 family of mitotic checkpoint genes (Davenport *et al.*, 1999); (3) EB1, a microtubule-associated protein that binds the tumor suppressor APC and targets it to microtubule tips (Askham *et al.*, 2000; Mimori-Kiyosue *et al.*, 2000); (4) AIM-1, an Aurora/Ipl1p-related serine/threonine kinase, that is required for cytokinesis (Terada *et al.*, 1998); (5,6) KRP1 and KRP2, members of the kinesin superfamily of motor proteins; (7) cdc2, a mitotic cyclin dependent kinase, and (8) Ki-67, a protein that localizes around mitotic chromosomes (Gerdes *et al.*, 1983) and is thought to be involved in mitotic chromosome organization.

The experiments described here are performed using cells re-entering the cell cycle after quiescence and under these experimental conditions the induction of these mitotic genes is detected already in S phase. It remains to be determined whether their expression is induced at this stage of the cell cycle also in cycling cells.

Additional studies are required to determine whether these mitotic genes are activated by E2Fs directly or indirectly. The latter possibility should be considered in view of the recent report by Muller *et al.* (2001) demonstrating that expression of a number of transcription factors is regulated by E2Fs.

The promoters of only two of these mitotic genes, cdc2 and SAK-a, have been identified (Hudson *et al.*,

2000; Yamamoto *et al.*, 1994). The promoter of the human *cdc2* gene contains an E2F-binding site and expression of the *cdc2* gene is regulated by E2F via this binding site (Tommasi and Pfeifer, 1995; Yamamoto *et al.*, 1994). Thus, *cdc2* is a direct E2F target gene. Our analysis of the murine SAK-a promoter demonstrates that it contains four putative E2F-binding sites suggesting that it too is a direct E2F target gene.

A possible role for E2F in M phase has been previously inferred by the E2F-dependent regulation of *cdc2* expression. E2F activity was shown to be required also for the accumulation of the mitotic cyclin, cyclin B1 (Lukas *et al.*, 1999). Furthermore, regulation of mitotic genes by E2F was recently reported also by Ishida *et al.* (2001). Of the E2F-regulated mitotic genes described here, only Ki-67 and *cdc2* were identified also by Ishida *et al.* (2001). Thus, our work identifies several novel E2F target genes that play a role in mitosis and it further supports the notion that E2F plays a role in the regulation of M phase entry and progression. Similarly, the list of E2F-regulated DNA replication genes identified by our screen (Table 1 and Kalma *et al.* (2001)) makes several significant additions to the present understanding of the relationship between E2F and DNA replication. Our data, together with those of Ishida *et al.* (2001), strongly suggest that E2Fs control cell cycle progression by regulating expression of both S phase genes and mitotic genes. In addition, our data imply that E2Fs regulate expression of genes involved in the response to DNA damage, thereby underscoring a novel function of E2F.

Materials and methods

Cell culture

Rat-1a fibroblasts that had stably integrated the p1093, p1093-E2F-1 or p1093-HA-E2F-3 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and G418 (500 μ g/ml). H1299 cells were grown in RPMI supplemented with 10% FCS. Cells were maintained at 37°C in a humidified 8% CO₂-containing atmosphere.

Cell-cycle flow cytometry assays

Bromodeoxyuridine (BrdU, final concentration 10 μ M) was added to cells 30 min prior to harvesting. Cells were trypsinized and fixed with 70% ethanol (-20°C). After fixation, cells were centrifuged for 5 min at 1200 r.p.m., and incubated for 30 min at room temperature in 1 ml of 2 M HCl/0.5% triton X-100. After recentrifugation, cells were washed with 1 ml of 0.1 M Na₂B₄O₇ (pH 8.5) and reacted with anti BrdU antibody (Becton Dickinson, 347580, 15 μ l per test) and then with Fluorescein isothiocyanate-conjugated anti-mouse antibody. Then cells were resuspended in PBS containing 5 μ g/ml propidium iodide for 30 min at room temperature. Fluorescence intensity was analysed using a Becton Dickinson flow cytometer.

Western blotting

Cells were lysed in lysis buffer (20 mM HEPES pH 7.8, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, phenylmethylsulfonyl fluoride (43 μ g/ml), aprotinin (17 μ g/ml), leupeptin (22 μ g/ml)). Equal amounts of protein from each lysate, as determined by Bradford assay, were resolved by electrophoresis in a SDS 10% polyacrylamide gel and transferred to a filter (Protran BA 85, S&S). Filter was incubated with an anti HA antibody (16B12, BAbCO) overnight after 2 h incubation in PBS with 0.05% Tween and 5% dry milk. Binding of the primary antibody was detected using an enhanced chemiluminescence kit (ECL Amersham).

DNA microarrays

Rat-1-MT-HA-E2F-3 and Rat-1-MT-E2F-1 cells were kept for 48 h in DMEM containing 0.1% FCS. Cells were treated with 100 μ M ZnCl₂ for 12 h, 14 h and 16 h or not treated and then cells were harvested. Total RNA was extracted using Tri Reagent method (Molecular Research Center) and double strand cDNA was synthesized by reverse transcription. Biotin-labeled cRNA was produced from the cDNA and used to probe Affymetrics Rat DNA microarray (Rat Genome U34A array). Hybridization and washes were performed using Affymetrics gene chip system according to the manufacturer's instructions.

RT-PCR

Reverse transcription-PCR (RT-PCR) was performed on total RNA prepared by the Tri Reagent method. 7.5 μ g of RNA were employed for cDNA synthesis using M-MLV reverse transcriptase (Promega, 200 u) and oligo dT (Pharmacia, 0.5 μ g). PCR was performed on 1 μ l of the 20 μ l cDNA sample. Below are indicated, respectively, the number of cycles, annealing temperature and the sequences of 5' and 3' primers used for each of the tested genes. For the gene encoding MSH2, 30 cycles, 57°C using 5'-CAGAGACAGGTTGGAGTTGGG and 5'-GCA-GCCAGAGACTGAGAGCC. For the gene encoding MSH6, 29 cycles, 57°C using 5'-GCTGTAAACGATACTGGACC and 5'-ACCATGCATGCCATATGTCC. For the gene encoding UNG, 28 cycles, 58°C using 5'-GGACCTAATCAAGCTCAC-GG and 5'-TCCGTGAACTGCTCCCAGCC. For the gene encoding PMS2, 31 cycles, 57°C using 5'-TCAGACAATG-GATGTGGGG and 5'-GGGGCAGCTGAACAAAAGG. For the gene encoding BUB1b, 29 cycles, 58°C using 5'-CCTGGTGTTCACAGTATCGC and 5'-AGGGAGAAGA-ACAGTTAGCC. For the gene encoding KRP2, 30 cycles, 58°C using 5'-TGCCAACCTCAATTCCTCCC and 5'-CATCTCA-GAGAGCTCAAGCC. For the gene encoding SAK-a, 32 cycles, 55°C using 5'-AGGAGGTGTGTGGAGC and 5'-TG-ACCCTCATTAGCATGG. For the gene encoding EB1, 28 cycles, 58°C using 5'-CAGTGAATGTGTACTCGACG and 5'-CCCATCTTCTGAAGCCG. For the gene encoding AIM-1, 30 cycles, 58°C using 5'-AGATTGGGCGTCTCTGGG and 5'-TCAATCATCTCTGGGGCAG. For the gene encoding ARPP-PO, 19 cycles, 58°C using 5'-GTGGGAGACAAA-TGTGG and 5'-CAGCTGCACATCGCTCAGG.

Isolation of human MSH2 promoter

An 852 bp fragment of the MSH2 promoter from -852 to -1 was generated by PCR using human genomic T98G DNA as template and 60 pm each from the 5' and 3' primers: 5'-CCCGGTACCCAAGTGATCCGCCACCTCG-3' and 5'-CCCAAGCTTGTCGAAACCTCCTCACCTCC-3'.

Plasmids

The luciferase reporter plasmid MSH2 (−852 to −1) Luc was constructed by subcloning the 852 bp *KpnI/HindIII* fragment of the MSH2 promoter in PGL2B-Luc. p1093-HA-E2F-3 was constructed by PCR.

The following plasmids have been previously described: pCMV- β -Gal (Lindeman *et al.*, 1997), pcDNAI-E2F-1, pcDNAI-HA-DP-1 (Krek *et al.*, 1993), pRcCMV-HA-E2F-1(1-363) (Hofmann *et al.*, 1996), pCMV-HA-DP-1(127–140) and pCMV-HA-DP-1(Δ 103–126) (Wu *et al.*, 1996).

Transfection assay

H1299 cells were transfected by the calcium phosphate method. Cell lysis, β -Gal, and luciferase assays were performed essentially as described (Lindeman *et al.*, 1997).

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