Individual promoter and intron p53-binding motifs from the rat Cyclin G1 promoter region support transcriptional activation by p53 but do not show co-operative activation

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Abstract The rat Cyclin G1 gene promoter contains one p53-binding motif upstream of the transcription start site, and a second motif downstream in the first intron. We have investigated the possibility that these motifs co-operate to permit high level promoter activation by p53. Although individual motifs supported p53-dependent, orientation-independent transcriptional activation, using reporter plasmids containing both motifs, we found no evidence for co-operative promoter activation either after co-transfection with human p53 expression plasmids, or after exposure of transfected cells to cisplatin and UV-radiation.

Key words: p53; Transcription; Cyclin G1; Promoter; Gene activation

1. Introduction

Many functions of the p53 tumour suppressor protein involve transcriptional activation of target genes (reviewed in [1]) requiring binding of p53 to a specific motif within the control regions of these genes [2–5]. Interestingly, the p21/WAF1 [6], IGFBP3 [7], TGFa [8], EGF receptor [9] and muscle creatine kinase (MCK) [10] genes contain multiple copies of this motif within their control regions. Though the functional significance of this arrangement is not yet clear, recent studies of the MCK promoter have suggested that multiple p53-binding sites may co-operate to allow high level, p53-dependent transactivation, moreover, this process caused looping-out of the promoter DNA sequence between the p53-binding motifs [10–13].

Cyclin G1 is a recently identified p53 target gene, which is induced after exposure to UV-radiation [14], γ-radiation [15] or actinomycin-D [16]. Promoter regions of the mouse, rat and human genes each possess two motifs with homology to the consensus p53-binding site [15,17–19]). One is approximately 250 bp upstream of the transcription start site (promoter p53-binding motif) and separated by about 500 bp from a second motif downstream and within the first intron (intron p53-binding motif). Previous studies have demonstrated that promoter and intron motifs from the rat gene can bind p53 [20] and that the promoter motif from the mouse and rat genes supports p53-dependent transactivation [15,21]. It is likely that the intron p53-binding motif has similar function, but at present there are no available data which have directly addressed this notion.

We have investigated the possibility that promoter and intron p53-binding sites from the Cyclin G1 promoter region may co-operate to permit high level, p53-dependent promoter activation. We find that promoter and intron p53-binding sites from the rat Cyclin G1 promoter region each support p53-dependent, orientation-independent, transcriptional activation. However, using reporter plasmids containing both motifs, we find no evidence for co-operative promoter activation either after co-transfection with human p53 expression plasmids, or after exposure of transfected cells to UV-radiation and cisplatin.

2. Materials and methods

2.1. Cell culture, transfection procedure and CAT assay

Mouse L929 fibroblasts were maintained in Eagle's modified essential medium (EMEM; Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Trace Biosciences, Sydney, Australia) and containing added L-glutamine (2 mM final concentration), penicillin/streptomycin (50 U/ml, Gibco, BRL) and fungizone (2.5 mg/ml, Trace Biosciences). Human bladder cancer cells UCRU-BL13/0 (BL13; [22]) were maintained in RPMI with the above supplements.

In each experiment, 5×10^5 L929 cells seeded into 6 cm petri dishes were transfected with 6 μg plasmid DNA and 9 μl of lipofectamine reagent (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) as described by the manufacturers. If the amount of reporter and p53 expression vector DNA was less than 6 μg, total DNA was maintained at 6 μg by addition of empty CMV vector DNA. The lipid/DNA complex was removed from cells after 5 h. Cells were harvested 18 h later and levels of CAT activity were measured as described [10]. A similar approach was used in experiments with BL13 cells except that cells were transfected with 3 μg total DNA. If the amount of reporter was less than 3 μg, total DNA was maintained at 3 μg by addition of empty CMV vector DNA. After 24 h, cells were exposed to 10 μg/ml cisplatin (cisplatinum (II) diamine dichloride, Sigma Chemical Company, Sydney, NSW) for 1 h or 100 J/m^2 UV-radiation (wavelength 254 nm using a Stratagene Stratalinker) before harvesting 24 h after treatment.

2.2. Plasmids

Construction of pCMVH6K (empty vector), and both pCMVtag wtp53 (human wtp53) and pCMVtag 1–323 (human p53 residues 1–323) which express p53 protein from the human cytomegalovirus early promoter/enhancer, has been described [13].

Reporter plasmids containing the promoter p53-binding motif from the rat Cyclin G1 gene were constructed as follows: rat Cyclin G1 promoter region sequences between −295 to −225 (P-sequence) relative to the proposed transcription start site [23], and containing the promoter p53-binding site between residues −272 to −251, were amplified by PCR from pGL32-Basic-super Cyclin G-luciferase (Zauberman, unpublished data) using a 5′ primer, 5′-AACCCTGCAGCAGGCCAATCTGGCTATTAGCAGCT-3′ (Primer 1) and a 3′ primer, 5′-AACCCTGCAGGCTGCGGAACTGAATGTTCAATGCC-3′ (Primer 2). After purification, the fragment was digested with *Pst*I, purified and ligated into the unique *Pst*I site within

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p-80MCKCAT [10,24] to form pPromCAT (5′-3′ in the Cyclin G1 sequence) or pmorPCAT (3′ to 5′ in the Cyclin G1 sequence) respectively. Orientation was verified by restriction enzyme analysis. This placed the Cyclin G1 promoter p53-binding site immediately 5′ to 87 bp of the proximal promoter sequence from the mouse muscle creatine kinase (MCK) gene. A similar approach was used to generate pIntronCAT (5′ to 3′) and pnortnICAT (3′ to 5′) each containing the intron p53-binding sequence from the rat Cyclin G1 gene. In this case, promoter sequence between +227 and +294 (I-sequence) containing the Cyclin G1 intron p53-binding site between residues +249 and +268 was amplified using a 5′ primer, 5′-AACCCTGCAGCCTCAGAGCCCTACACTCCCGAACG-3′ (Primer 3) and a 3′ primer, 5′-AACCCTGCAGCGCTCCCACCGCGCCTCTCTAGGAG-3′ (Primer 4).

Plasmids pPICAT and prPICAT containing both rat Cyclin G1 p53-binding motifs separated by 3 kb of p-80MCKCAT vector sequence, were generated by inserting the Cyclin G P-sequence into the unique *Kpn*I site within pIntronCAT. Primers were used as described above (Primers 1 and 2) but with *Kpn*I rather than *Pst*I restriction enzyme sites incorporated into the ends.

Plasmid pPEIGCAT (containing both rat Cyclin G1 gene p53-binding sites separated by the normal 500 bp of Cyclin G1 promoter sequence) was constructed by amplifying promoter sequences between −295 and +294 using Primers 1 and 4 described above. After *Pst*I digestion and purification, the DNA fragment was cloned into the *Pst*I site within the polylinker sequence of p-80MCKCAT [24]. Correct orientation of this fragment was confirmed by restriction enzyme analysis. A similar approach was used to generate pEIGCAT (containing promoter sequences between −1 and +294) and pPEGCAT (containing promoter sequences between −295 and +248). For pEIGCAT, amplification used a 5′ primer, 5′-AACCCTGCAGACCGCACGCTGAACCGGAGGAAGGC-3′ and Primer 4 described above, whilst for pPEGCAT, Primer 1 from above and a 3′ primer, 5′-AACCCTGCAGTCGGGAGTGTAGGGCTCTGAGGTCC-3′ were used.

MCK reporter plasmids containing distal and proximal (pD2800PMCKCAT), proximal only (p2800PMCKCAT), distal only (pD3300MCKCAT) or no (p2800MCKCAT) p53-binding sites have been described previously [13].

2.3. PCR conditions

P- and I-sequences were amplified with a Hybaid Touchdown Thermocycler in a 50 μl reaction containing 1 μl template (50 ng/μl), 1 μl each primer (20 pmol/μl), 4 μl dNTP mix (25 mM/μl), 5 μl 10×DeepVent reaction buffer and 0.5 μl DeepVent enzyme (New England Biolabs Inc., Beverley, MD). Hotstarting at 94°C, 1 min before enzyme addition then 72°C, 30 s, was followed by a cycle of 94°C, 1 min; 60°C, 30 s; 72°C, 30 s, and 40 cycles of 94°C, 1 min; 72°C, 30 s. A final step was 72°C 10 min. Other rat Cyclin G1 promoter region fragments were amplified under similar conditions but with reaction mixtures containing 4 μl 25 mM $MgCl_2$, the initial annealing temperature was 55°C, and reactions were held at 94°C or 72°C for 30 s or 3.0 min respectively.

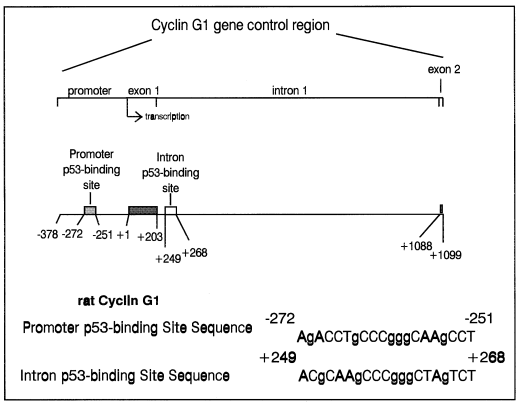

Fig. 1. Schematic illustration of the rat Cyclin G1 promoter region and p53-binding elements. Numbers indicate nucleotide position relative to the proposed transcription start site [23].

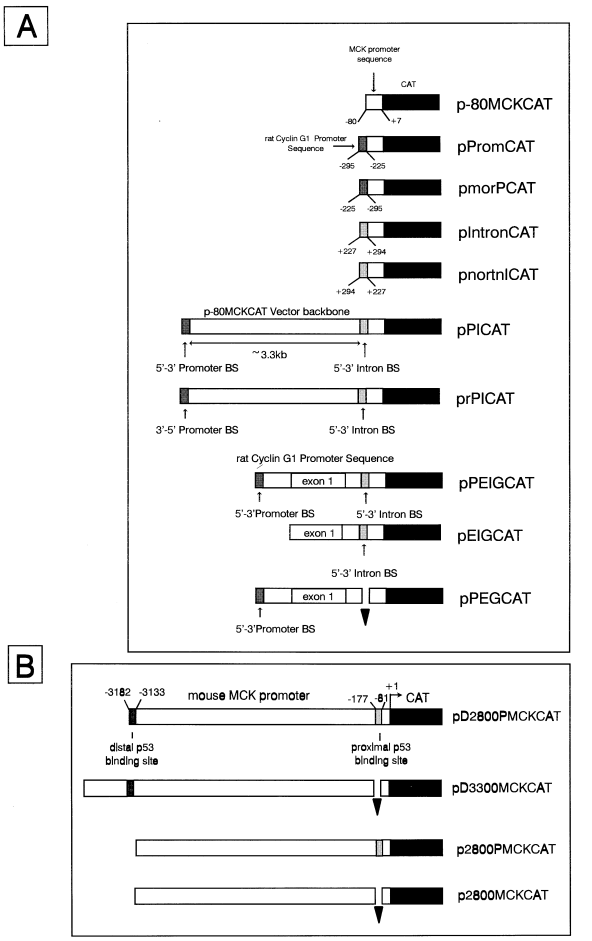

Fig. 2. Schematic illustration of Cyclin G1 (A) and MCK (B) reporter plasmids used in this study. Numbers refer to the nucleotide sequence relative to the transcription start site for each promoter.

3. Results

3.1. Individual p53-binding motifs from the rat Cyclin G1 promoter support transcriptional activation by p53

The rat Cyclin G1 gene promoter region contains two motifs with homology to the consensus p53-binding sequence (Fig. 1) which bind p53 with approximately equal affinity [20]. Promoter sequences between −295 to −225 (with the promoter p53-binding site from residues −272 to −251) and +227 to +294 (with the intron p53-binding site from residues +249 and +268) were amplified and cloned in forward (5′-3′, pPromCAT and pIntronCAT) and reverse (3′-5′, pmorPCAT and pnortnICAT) orientations into p-80MCKCAT, such that each motif was upstream and adjacent to a short fragment of the muscle creatine kinase (MCK) promoter (Fig. 2A). These plasmids were co-transfected into L929 cells (possessing wild-type endogenous p53) with either control plasmid (pCMVH6K), or plasmid expressing wild-type human p53 (pCMVtag wtp53). All reporters having p53-binding motifs showed transcriptional activation by p53 (Fig. 3), with those containing the promoter motif activated to a greater extent than those with the intron motif. Maximal activation (10–15× for pPromCAT/pmorPCAT, and 3–4× for pIntronCAT/pnortnICAT) was seen with 0.5–2 μg pCMVtag wtp53. At higher plasmid doses (4–5 μg), we saw reduced promoter

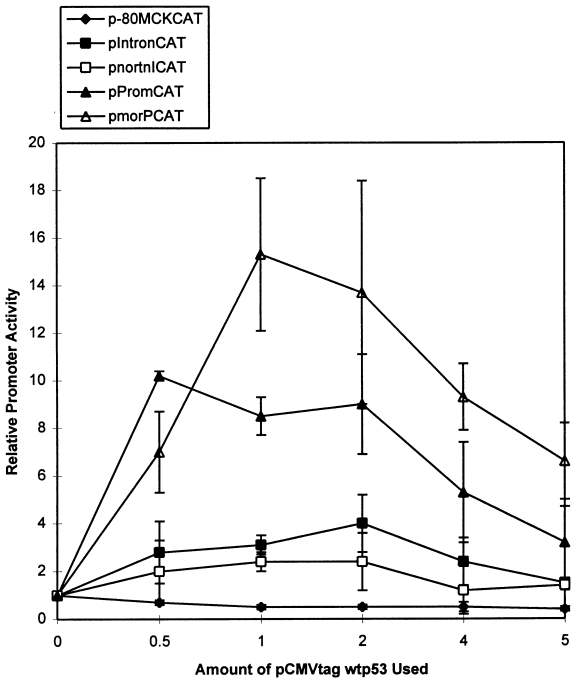

Fig. 3. Individual p53-binding motifs from the Cyclin G1 promoter region permit p53-dependent, orientation-independent transcriptional activation. L929 cells were transfected with 1 μg of each reporter plasmid and increasing concentrations of pCMVtag wtp53 as described in Section 2. Results presented show the mean ± S.E. of at least three independent experiments.

activation, possibly reflecting sequestration by p53 of protein factors required for transcription. Without p53-binding motifs (p-80MCKCAT), p53 had no effect on transcription (Fig. 3).

We conclude that promoter and intron p53-binding motifs from the rat Cyclin G1 promoter region each mediate wt human p53-dependent, orientation-independent transactivation.

3.2. Rat Cyclin G1 p53-binding sites fail to show co-operative activation

To determine if Cyclin G1 p53-binding sites might co-operate to permit high level synergistic promoter activation by p53, reporter plasmids were generated in which promoter and intron p53-binding motifs were separated by 3.3 kb of vector sequence (pPICAT and prPICAT; Fig. 2A). Activation of these promoters by wtp53, or a truncated p53 protein which is monomeric and shows reduced ability to activate transcription from individual p53-binding sites (pCMV 1–323; [13,25]), was then compared to activation of promoters with individual p53-binding motifs. Chances of detecting co-operative activation were maximised by using transfection conditions giving minimal, reproducible activation of promoters with single p53-binding motifs (see Fig. 3). As controls, we used MCK reporter plasmids in which both MCK p53-binding motifs were present (pD2800PMCKCAT) or from which either distal (p2800PMCKCAT), proximal (pD3300MCKCAT) motif or both motifs (p2800MCKCAT) had been deleted (Fig. 2B; [13]). Results illustrated in Fig. 4A confirmed that in L929 cells, wtp53 induced high level, co-operative activation of pD2800PMCKCAT (13.5×) compared with p2800PMCKCAT (3.5×) or pD3300MCKCAT (1.3×). Cyclin G1 reporter plasmids pPICAT and prPICAT were also activated by wtp53 (Fig. 4B) but rather than high level induction consistent with co-operation between p53-binding sites, activation was only 5–6× and similar to that obtained from promoters with single p53-binding motifs. In all cases, the truncated p53 protein was less effective than the wt protein.

In further studies, we obtained similar results even if the amount of transfected wtp53 plasmid was increased to maximum permissible levels (from 0.5 μg to 5.5 μg plasmid; data not shown), suggesting that lack of co-operation was not due to limiting amounts of transfected p53 protein. In addition, we were unable to detect co-operative promoter activation in experiments with monkey CV1 cells which also contain low levels of endogenous wtp53 and which have been previously used to demonstrate co-operative activation of the MCK promoter by p53 [25].

3.3. Cyclin G1 promoter sequences between p53-binding motifs do not permit co-operative promoter activation by p53

Conceivably, the absence of co-operative promoter activation using pPICAT and prPICAT might have been due to the absence of normal Cyclin G1 promoter sequences between p53-binding motifs. To address this possibility, we constructed reporter plasmids (Fig. 2A) having Cyclin G1 promoter and

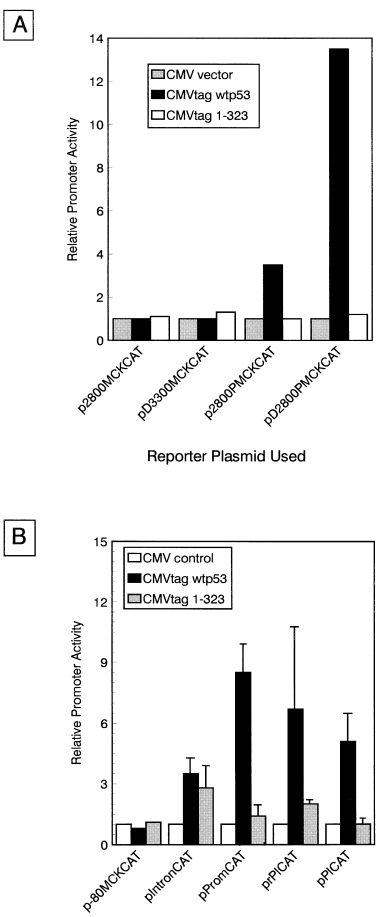

Fig. 4. Co-operative activation by p53 in promoters with MCK (A) but not Cyclin G1 (B) p53-binding sites. L929 cells were transfected with 1 mg of the indicated reporter plasmids and 0.5 μg of either pCMVtag wtp53 or pCMVtag 1–323 as described in Section 2. Results presented show the mean of two (A) or mean ± S.D. of three (B) independent experiments.

intron p53-binding motifs separated by the normal 500 bp of promoter sequence (pPEIGCAT) or from which either the promoter (pEIGCAT) or the intron (pPEGCAT) motif was deleted. These plasmids were co-transfected into L929 cells along with control vector and pCMVtag wtp53. Results shown in Fig. 5 indicated that pEIGCAT and pPEGCAT were each activated ~4× by wtp53. Consistent with our results above however, no evidence for co-operative promoter activation was obtained using pEIGCAT, rather activation (3.5×) was again similar to that obtained from promoters with single p53-binding motifs.

Taking these data into consideration with the results described in the previous section, we conclude that promoter and intron p53-binding motifs from the rat Cyclin G1 promoter do not support co-operative promoter activation by human wtp53.

3.4. Co-operation between Cyclin G1 p53-binding sites does not occur after exposure to cisplatin or UV-radiation

Cyclin G1 is induced after exposure of cells to UV-radiation [14], γ-radiation [15] or actinomycin-D [16]. We wished to determine if co-operative promoter activation involving Cyclin G1 p53-binding motifs might require specific cellular conditions induced by DNA damage. In spite of considerable effort, in initial experiments we were unable to demonstrate p53-dependent induction of any Cyclin G1 reporter plasmid in transfected L929 cells exposed to cisplatin or UV. As an alternative approach, we used BL13 human bladder cancer cells which contain wt human p53 [26] and which have been shown to exhibit increased transcription of a transfected p53-dependent reporter plasmid (pD2800PMCKCAT) after exposure to cisplatin [27]. We confirmed that maximal levels of endogenous p53 induction were achieved by 24 h after exposure of BL13 cells to either 10 μg/ml cisplatin or 100 J/m^2 UV-radiation (data not shown). The ability of UV and cisplatin to induce activation of pPEIGCAT, pEIGCAT and pPEGCAT in transfected cells was then compared. The positive control for these experiments was pD2800PMCKCAT, which was activated by both cisplatin (~6.5×) and UV (~3×) relative to control, untreated cells (Fig. 6). UV-radiation clearly activated transcription from all Cyclin G1 reporter plasmids but the level of activation was similar for all plasmids (~2.5–3×, Fig. 6). Cisplatin also induced pPEIGCAT and pEIGCAT to similar levels (~2.5×) but in the absence of the intron-binding site (pPEGCAT) activation was almost completely abolished.

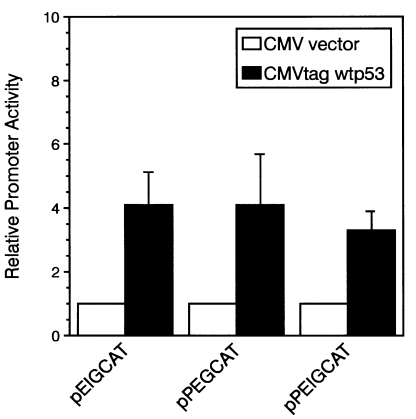

Fig. 5. Absence of p53-dependent, co-operative promoter activation by deletion analysis of Cyclin G1 reporter plasmids. L929 cells were transfected with 2 μg of each reporter plasmid and 2.0 μg of pCMVtag wtp53 as described in Section 2. Results presented show the mean ± S.D. of three independent experiments.

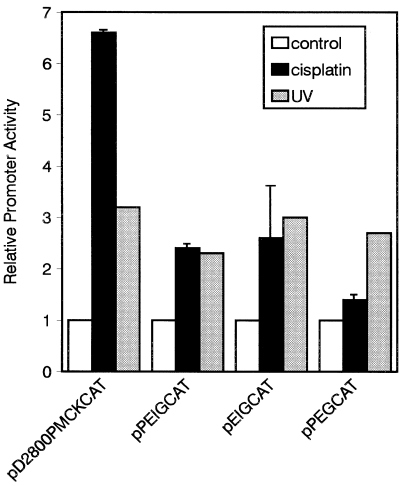

Fig. 6. Cyclin G1 reporter plasmids do not show co-operative promoter activation in response to cisplatin or UV. BL13 cells were transfected with 3 μg of pD2800PMCKCAT or 2 μg of Cyclin G1 reporter plasmids and exposed to 10 μg/ml cisplatin or 100 J/m^2 UV as detailed in Section 2. Results presented show the mean of two (UV) or mean ± S.D. of three (cisplatin) independent experiments.

We conclude that promoter and intron p53-binding motifs from the rat Cyclin G1 promoter do not support co-operative promoter activation by human wtp53 after exposure of cells to cisplatin or UVC.

4. Discussion

Studies using either a model promoter or the MCK gene promoter have suggested that multiple p53-binding motifs within p53 target gene promoters may co-operate to permit high level, synergistic activation by p53 [11–13]. We have extended these studies to examine the rat Cyclin G1 gene promoter region which also contains two p53-binding motifs [15,17–19].

Although individual binding sites supported activation of a heterologous promoter by p53, we were unable to demonstrate co-operative activation using (i) promoters with p53-binding motifs separated by heterologous promoter sequences, (ii) deletion analysis of promoters with motifs separated by the normal Cyclin G promoter sequence, (iii) promoters where the relative orientation of each element was inverted. Whilst we cannot rule out the possibility of promoter sequences outside the studied region being required, we suggest that the simplest explanation for these data is that the two p53-binding motifs from the Cyclin G promoter region do not co-operate in p53-dependent promoter activation.

Cyclin G1 is induced after exposure of cells to a variety of DNA damaging agents [14–16]. In addition, studies of p53 phosphorylation have suggested that different cellular environments have a major influence on the response of p53 target genes, including Cyclin G1, to p53 [21]. As a consequence, we investigated the possibility that DNA damaging agents might induce cellular changes which permitted co-operative pro-

moter activation involving Cyclin G1 p53-binding motifs. However, though able to show modest promoter activation after exposing transfected cells to both cisplatin and UV, we failed to observe high level, co-operative promoter activation after treatment with either agent.

The two p53-binding motifs in the Cyclin G1 promoter are conserved between human, rat and mouse genes [19], suggesting that this organisation is functionally important. If not involved in co-operative promoter activation by p53, then how might two different p53-binding motifs contribute to regulation of Cyclin G1 expression by p53? Although the motifs bind p53 with equal affinity [20], one possibility is that each may respond differently to p53. Consistent with this notion, in transfection experiments, we observed that the promoter motif clearly permitted greater transcriptional activation of a heterologous promoter than the intron motif, and obtained some preliminary data to suggest that the intron motif is responsible for the ability of UV to induce promoter activation. Moreover, in a recent study with reporter plasmids containing either both motifs or the promoter motif only, Lohrum and Scheitmann provided evidence that the intron motif was responsible for the repression of Cyclin G1 promoter activity after okadaic acid-induced hyperphosphorylation of p53 [21]. At present, however, information on the biological role of Cyclin G1 is limited. As a consequence, the physiological basis for activation of the Cyclin G1 gene by p53 is not clear and the functional importance of two p53-binding sites within the promoter region remains to be determined.

References

[1] Ko, L.J. and Prives, C. (1996) Genes Dev. 10, 1–15.
[2] Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C. and Vogelstein, B. (1991) Science 252, 1708–1711.
[3] El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W. and Vogelstein, B. (1992) Nature Genet. 1, 45–49.
[4] Funk, W.D., Pak, D.T., Karas, K.H., Wright, W.E. and Shay, J.W. (1992) Mol. Cell. Biol. 12, 2866–2871.
[5] Halazonetis, T.D., Davish, J. and Kandil, A.N. (1993) EMBO J. 12, 1021–1028.
[6] El-Deiry, W.S., Tokino, T., Waldman, T., Oliner, J.D., Velculesco, V.E., Burrell, M., Hill, D.E., Healy, E., Rees, J.C. and Hamilton, S.R. et al. (1995) Cancer Res. 55, 2910–2919.
[7] Buckbinder, L., Talbott, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Seizinger, B.R. and Kley, N. (1995) Nature 377, 646–649.
[8] Shin, T.H., Paterson, A.J. and Kudlow, J.E. (1995) Mol. Cell. Biol. 15, 4694–4701.
[9] Sheikh, M.S., carrier, F., Johnson, A.C., Ogdon, S.E. and Fornace Jr., A.J. (1997) Oncogene 15, 1095–1101.
[10] Jackson, P., Shield, M., Buskin, J., Hawkes, S., Reed S., M., Perrem, K., Hauschka, S.D. and Braithwaite, A.W. (1995) Gene Express. 5, 19–33.
[11] Stenger, J.E., Tegtmeyer, P., Mayr, G.A., Reed, M., Wang, Y., Wang, P., Hough, P.V.C. and Mastrangelo, I. (1994) EMBO J. 13, 6011–6020.
[12] Jackson, P. and Yardley, G. (1997) FEBS Lett. 406, 271–274.
[13] Jackson, P., Mastrangelo, I., Reed, M., Tegtmeyer, P., Yardley, G. and Barrett, J. (1998) Oncogene 16, 283–292.
[14] Rosen, C.F., Poon, R. and Drucker, D.J. (1995) Am. J. Pathol. 268, C846–855.
[15] Okamoto, K. and Beach, D. (1994) EMBO J. 13, 4816–4822.
[16] Bates, S., Rowan, S. and Vousden, K.H. (1996) Oncogene 13, 1103–1109.
[17] Zauberman, A., Lupo, A. and Oren, M. (1995) Oncogene 10, 2361–2366.
[18] Endo, Y., Fujita, T., Tamura, K., Tsuruga, H. and Nojima, H. (1996) Genomics 38, 92–95.
[19] Jensen, M.R., Factor, V.M., Zimonjic, D.B., Miller, M.J., Keck, C.L. and Thorgeirsson, S.S. (1997) Genomics 45, 297–303.
[20] Zauberman et al., unpublished data.
[21] Lohrum, M. and Scheidtmann, K.H. (1996) Oncogene 13, 2527–2539.
[22] Russell, P.J., Wass, J., Lukeis, R., Garson, M., Jelbart, M., Wills, E., Phillips, J., Brown, J., Carrington, N., Vincent, P.C. and Raghavan, D. (1989) Int. J. Cancer 44, 276–285.
[23] Tamura, K., Kanaoka, Y., Jinno, S., Nagata, A., Ogiso, Y., Shimizu, K., Hayakawa, T., Hojima, H. and Okayama, H. (1993) Oncogene 8, 2113–2118.
[24] Jaynes, J.B., Johnson, J.E., Buskin, J.N., Gartside, C.L. and Hauschka, S.D. (1988) Mol. Cell. Biol. 8, 62–70.
[25] Wang, Y., Schwedes, J.F., Parks, D., Mann, K. and Tegtmeyer, P. (1995) Mol. Cell. Biol. 15, 2157–2165.
[26] Ribeiro, J.C., Hanley, J.R. and Russell, P.J. (1996) Oncogene 13, 1269–1278.
[27] Riberio, J.C. (1998) PhD Thesis, The University of New South Wales, Sydney, Australia.