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Transgenic mouse model for studying the transcriptional activity of the p53 protein: age- and tissue-dependent changes in radiation-induced activation during embryogenesis

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The p53 tumor suppressor protein is a sequence-specific transcriptional activator of target genes. Exposure of cells to DNA damage results in accumulation of biochemically active p53, with consequent activation of p53-responsive promoters. In order to study how the transcriptional activity of the p53 protein is regulated *in vivo*, a transgenic mouse strain was generated. These mice harbor the p53-dependent promoter of the *mdm2* gene, fused to a *lacZ* reporter gene. Induction of *lacZ* activity by DNA damage (ionizing radiation) was monitored in embryos of different p53 genotypes. The transgenic promoter was substantially activated *in vivo* following irradiation; activation required functional p53. The activation pattern became more restricted with increasing embryo age, as well as with the state of differentiation of a given tissue. Generally, maximal p53 activation occurred in rapidly proliferating, relatively less differentiated cells. A striking extent of haploinsufficiency was revealed—induction of promoter activity was far less efficient in mice carrying only one wild-type p53 allele. This suggests that normal levels of cellular p53 are limiting, and any further reduction already compromises the p53 response significantly. Thus, the activation potential of p53 is tightly controlled *in vivo*, both spatially and temporally, and an important element in this control is the presence of limiting basal levels of activatable p53.

Keywords: embryogenesis/*mdm2*/p53/transcriptional activation/transgenics

Introduction

The p53 protein is the product of a tumor suppressor gene, frequently mutated in human cancer (for recent reviews on p53, see Haffner and Oren, 1995; Gottlieb and Oren, 1996; Jacks and Weinberg, 1996; Ko and Prives, 1996). These mutations are believed to inactivate one or more biochemical functions of the wild-type (wt) p53 protein, functions which otherwise may interfere with tumor development.

The best characterized, and probably most important, biochemical function of wt p53 is the sequence-specific transactivation (SST) of target genes. The p53 protein can bind to specific sites within DNA and induce the transcription of genes residing in the vicinity of such p53-binding sites. A 20 bp consensus sequence for p53 binding has been defined (El-Deiry *et al.*, 1992; Funk *et al.*, 1992). Typical natural p53 binding sites, which drive p53-dependent expression of physiological target genes, usually diverge from this consensus by one or a few nucleotides.

In normal cells, p53 is believed to be biochemically latent (Hupp *et al.*, 1995). In addition, p53 is a very labile protein (Oren *et al.*, 1981; Rogel *et al.*, 1985) and, consequently, its steady-state levels are usually extremely low. However, in response to appropriate signals, p53 can become both stabilized and biochemically activated, resulting in a prominent increase in overall cellular levels of active p53. The best known inducer of p53 is DNA damage (Kastan *et al.*, 1991, 1992; Lu and Lane, 1993; Zhan *et al.*, 1993; Hupp *et al.*, 1995), although other signals, including hypoxia (Graeber *et al.*, 1996) and ribonucleotide depletion (Linke *et al.*, 1996), can also have similar effects. Activation of p53 in response to DNA damage is believed to prevent the propagation of cells with potentially dangerous genetic lesions (Lane, 1992). This preventive effect of p53 can be exerted through arresting cell cycle progression transiently, so that the damaged DNA can be repaired properly before the cell resumes proliferation. Alternatively, p53 induction may permanently remove damaged cells from the replicative pool, either through triggering apoptotic cell death or through imposing a permanent G_1 arrest.

The involvement of p53 in developmental processes has been studied by several complementary approaches. Mice lacking p53 ('p53 knock-out') appear to undergo perfectly normal development, although eventually succumbing to a variety of malignancies at a relatively early age (Donehower *et al.*, 1992). However, more recent work has identified a spectrum of developmental defects in a fraction of p53 null mice; these range from a failure in neural tube closure, resulting in exencephaly and embryonic death, to various less severe abnormalities (Armstrong *et al.*, 1995; Sah *et al.*, 1995). Thus, p53 may indeed have a role in normal development (Rotter *et al.*, 1994), but other genes may often cover up for its absence. The observed defects might reflect rare situations where p53 function cannot be fully substituted, as may be the case during spermatogenesis (Rotter *et al.*, 1993). Alternatively, p53 may be important only in embryos undergoing accidental or induced damage. Indeed, p53 can suppress radiation-induced teratogenesis, via a mechanism involving p53-mediated apoptosis (Norimura *et al.*, 1996).

In situ hybridization analysis has revealed p53 mRNA expression in all cells of the embryo up to embryonic day 10.5 (E10.5) (Schmid *et al.*, 1991). Later in development, expression of p53 mRNA becomes more heterogeneous, but high levels are still seen in many tissues. Yet, since p53 is subject to elaborate post-translational regulation,

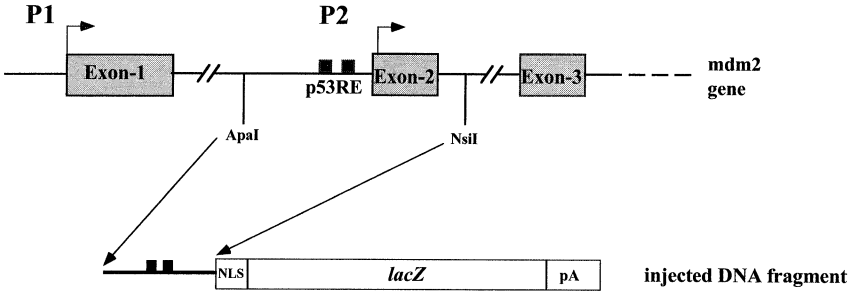

Fig. 1. Schematic diagram of the murine *mdm2* gene region including the intronic P2 promoter, and of the DNA fragment used for oocyte microinjection. The latter DNA fragment contains the indicated *Apa*I–*Nsi*I segment of the *mdm2* gene as well as a *lacZ* gene derived from plasmid pPD46.21 (see Materials and methods). Abbreviations: P1, p53-independent (constitutive) *mdm2* promoter; P2, p53-dependent intronic promoter (Juven *et al.*, 1993); p53RE, p53-responsive elements, represented by black boxes; NLS, nuclear localization signal, derived from SV40 large T antigen; pA, polyadenylation signal, derived from the SV40 early region. Exon numbers refer to transcripts initiated at the constitutive P1 promoter (Montes de Oca Luna *et al.*, 1996). Actual transcription start sites for P1 and P2 are indicated by arrows (see Barak *et al.*, 1994).

abundant mRNA does not necessarily imply abundant protein, and certainly not high p53 activity. This is exemplified by embryonal carcinoma (EC) cells. These cells, very similar to those of the early normal embryo, express ample p53 mRNA and protein (Oren *et al.*, 1982; Reich *et al.*, 1983); moreover, that p53 is wild-type by sequence (Lutzker and Levine, 1996; Pennica *et al.*, 1984). However, this p53 is practically devoid of SST activity (Lutzker and Levine, 1996), becoming active only when EC cells are either induced to differentiate *in vitro* or subjected to DNA damage (Lutzker and Levine, 1996). Hence, quantitative analysis of p53 mRNA, or even protein, is not a reliable measure of p53 activity.

To study the *in vivo* regulation of p53 function, particularly in response to stress signals activating the p53 pathway, we generated a transgenic mouse strain carrying a *lacZ* reporter gene under a p53-responsive promoter. We used the intronic promoter of the mouse *mdm2* gene which is a physiological target for transcriptional activation by wt p53. We describe here the effects of radiation on the activity of this promoter during embryogenesis. The data imply that the ability to mount an effective p53 SST response is greatly dependent on tissue type and developmental stage. In general, it becomes gradually more restricted with embryo maturation, and in cells relatively more advanced in differentiation. In addition, at least for the *mdm2* promoter, there is a striking *in vivo* p53 haploinsufficiency, resulting in a much weaker induction of p53-specific SST in embryos containing only one wt p53 allele. Different tissues exhibit non-identical degrees of haploinsufficiency. Hence, critical thresholds for activation of p53 target genes may vary with cell type.

Results

Generation of transgenic mice carrying an mdm2 promoter–lacZ fusion

In order to study the *in vivo* regulation of p53-mediated SST, a transgenic mouse model was developed. To that end, the p53-dependent intronic promoter of the murine *mdm2* gene (P2; Barak *et al.*, 1994) was placed in front of the bacterial β-galactosidase (*lacZ*) gene, acting as a reporter for transcriptional activity of the promoter. This particular promoter was chosen because it represents a well-characterized physiological target for transactivation by p53 (Juven *et al.*, 1993; Wu *et al.*, 1993; Barak *et al.*, 1994). Importantly, transcription from this promoter in at least three different cell types, fibroblasts and myeloid cells harboring a temperature-sensitive p53, and wt p53-expressing lymphoma cells exposed to ionizing radiation (IR), was shown by RNase protection to be strictly dependent on the presence of activated p53 (Barak *et al.*, 1994). This is unlike the p21 promoter, which can exhibit significant activity even in the absence of functional p53 (Michieli *et al.*, 1994; El-Deiry *et al.*, 1995; Halevy *et al.*, 1995; Parker *et al.*, 1995; Zhang *et al.*, 1995; Tanaka *et al.*, 1996), and unlike the constitutive P1 promoter of *mdm2*, which is essentially p53 independent (Barak *et al.*, 1994; Montes de Oca Luna *et al.*, 1996).

The design of the reporter construct, containing the *mdm2* P2 promoter within a 0.4 kb *Apa*I–*Nsi*I fragment (Juven *et al.*, 1993), is shown in Figure 1. This construct was microinjected into fertilized oocytes to generate transgenic mice. Positive founders were identified by Southern blot analysis. Each founder was then mated to a non-transgenic mouse, and expression of the *lacZ* transgene was monitored in irradiated E10.5 F1 embryos. Of six founders which transmitted the transgene, the progeny of two (#9 and #20) expressed detectable *lacZ* activity. The staining patterns of irradiated E10.5 embryos were essentially identical in both strains, except that staining intensity was substantially stronger in #20. Southern blot hybridization revealed that founders #20 and #9 harbored ~20 and five copies of the transgene, respectively (data not shown). Strain #20 was subjected to detailed analysis, as described below.

Activation of a p53-responsive promoter by DNA damage is temporally and spatially regulated during embryogenesis

A transgenic male was mated with non-transgenic females. The females were sacrificed at different times post-coitum (p.c.), and embryos were subjected to whole mount staining with X-Gal substrate to visualize *lacZ* activity. Weak positive staining could be observed in E8.5, E10.5 and E12.5 transgenic embryos (Figure 2A, C and E, respectively). At E8.5, there was staining in the branchial arches, in the tail bud and in the periphery of the somites; analysis of sectioned material revealed that the staining in the tail bud is superficial (data not shown). In E10.5 embryos, the clearest staining was seen in the branchial arches, the tips of the limb buds, the midbrain–hindbrain boundary, a

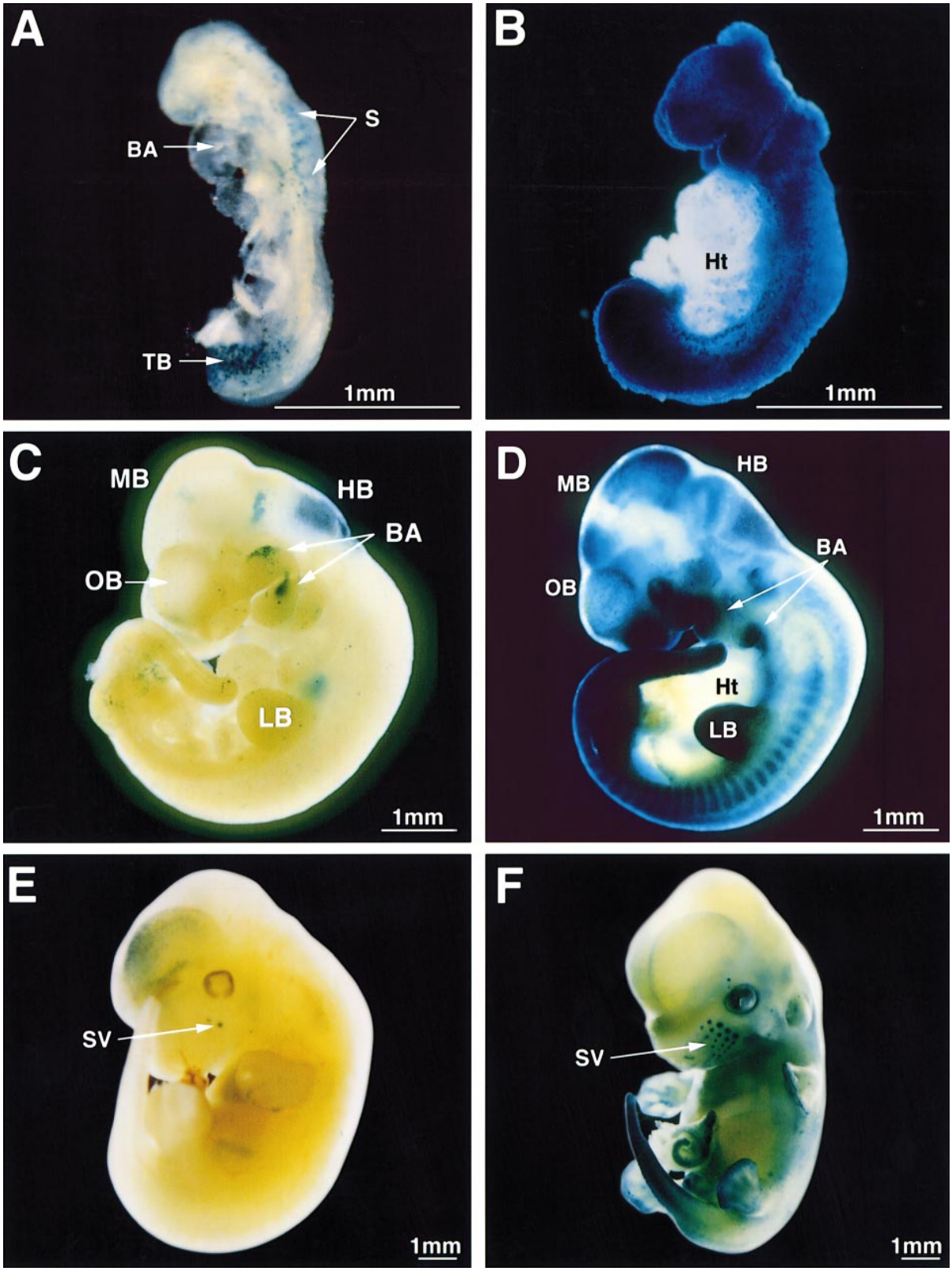

Fig. 2. Whole mount *lacZ* staining of embryos at different days of gestation. (**A**) and (**B**) E8.5, (**C**) and (**D**) E10.5 and (**E**) and (**F**) E12.5. Staining was performed either with (B, D and F) or without (A, C and E) prior exposure to 5 Gy of γ radiation. Incubation with the X-Gal substrate was for 4 h. The embryos presented in this figure were obtained from non-transgenic CB6/F1 females that had been mated with a transgenic male. Abbreviations: BA, branchial arches; HB, hindbrain; Ht, heart; LB, limb bud; MB, midbrain; OB, olfactory bulbs; S, somites; SV, sensory vibrissae; TB, tail bud.

small additional zone within the midbrain and the anterior part of the olfactory bulbs (Figure 2C). At E12.5, the pattern became more complex; significant staining could be observed also in the forebrain (in the cortex) and in the hair follicles of the sensory vibrissae (Figure 2E). It is of note that positive staining in the brain and hair follicles of unirradiated embryos, starting at E12, was also observed in transgenic mice in which *lacZ* expression is driven by a different, chimeric p53-responsive promoter (see accompanying paper by Komarova *et al.*). Detectable *lacZ* activity in our mice was dependent on the presence of the transgene; no staining could be detected in non-transgenic E8.5 and E10.5 embryos, and while some staining was observed at E12.5, this did not coincide with any of the major sites of *lacZ* activity shown in Figure 2E (data not shown).

The SST activity of p53 is known to be stimulated by DNA damage (Kastan *et al.*, 1992; Lu and Lane, 1993; Zhan *et al.*, 1993, 1994; Barak *et al.*, 1994; Dulic *et al.*, 1994; Gottlieb *et al.*, 1996). To determine whether this could also be demonstrated *in vivo* in our transgenic mice, pregnant females were subjected to whole body IR (5 Gy) 3 h prior to embryo isolation and staining. As can be clearly seen in Figure 2B, D and F, *lacZ* expression increased dramatically in these embryos following radiation exposure. This implies that the *mdm2* P2 promoter retains its p53 responsiveness in the transgenic mice, a conclusion further corroborated by the analysis of p53 null *lacZ* transgenic embryos (see below). These mice can thus be utilized for studying the pattern of transcriptional activity of endogenous p53 in animals exposed to DNA damage.

Intensive *lacZ* staining following irradiation could be seen at sites displaying constitutive basal expression in the untreated controls, as well as sites where no basal expression was detectable. In sites of the first type, radiation exposure caused a further increase in staining. Induction of *lacZ* staining, albeit to sub-maximal levels, could already be seen in E10.5 embryos as early as 1 h after irradiation (data not shown). This rapid induction is consistent with the notion that high SST activity is mediated by the activation of latent p53 protein, rather than the triggering of transcription from the p53 gene.

The data in Figure 2 demonstrate that the p53 response to DNA damage varies greatly among different tissues, as well as between different stages of development. In general, the older the embryo, the weaker and more tissue type restricted is the responsiveness of p53 to DNA damage. At E8.5, the entire embryo turns blue after irradiation, the only visible exception being the primitive heart (Figure 2B). On the other hand, at E10.5 the pattern of induction becomes more selective (Figure 2D). All three brain vesicles display positive staining at this stage. The branchial arches, as well as the maxillary areas, are strongly stained, and so are the limb buds. The somites and the tail bud display a gradient of staining, increasing towards the posterior end. In contrast, in the anterior portion of the trunk, *lacZ* activity is conspicuously weaker. Finally, similarly to E8.5, no staining is seen in the heart, suggesting that this tissue either makes no p53 protein, or contains latent protein which cannot be activated by radiation-induced DNA damage. It is of note that although p53 protein can be detected by immunohistochemistry in the heart of irradiated embryos, this tissue does not undergo any radiation-induced apoptosis (Wubah *et al.*, 1996; D.MacCallum and P.Hall, personal communication).

One of the best examples for age-dependent restriction of p53 SST activation in the embryo is the developing neural tube, which is uniformly and intensely stained in irradiated E8.5 embryos (Figure 3A), while staining becomes confined to a limited subset of cells at E10.5 (Figure 3B). Moreover, a strip lacking detectable *lacZ* induction is visible in the midbrain, within the roof of the third ventricle, at E10.5 (Figures 2D and 3C). The tissue-specific differences in p53 SST induction are also evident in an eosin-stained paraffin section (Figure 3D), which in addition shows clear positive expression of the transgene in the Rathke's pouch, and in contrast no detectable expression in parts of the midbrain, and particularly the ventral midbrain (small arrow in Figure 3D). Thus, at this stage, some neuronal tissues are already selectively losing their ability to activate p53 SST function in response to DNA damage.

At E12.5, there is a further increase in selectivity of p53 SST induction (Figure 2F). Strong expression of *lacZ* is evident in the eye, the sensory vibrissae and the tail; notably, the developing eye is a site of marked p53 protein accumulation and apoptosis in irradiated embryos (Wubah *et al.*, 1996; D.MacCallum and P.Hall, personal communication). The signal in the limb buds becomes more specialized, and is evident mainly in the apical ectodermal ridge and in the prospective fingers and toes. Here too, an anterior–posterior gradient is visible along the tail, with the most intense staining seen in the posterior part. It is well established that the axial structures differentiate in an anterior to posterior succession, such that the posterior paraxial mesoderm may still be in an unsegmented state at the time when the thoracic somites have already differentiated to their mature components. We see strong induction of p53 SST activity in the posterior, less differentiated portion of the axis, as well as in the limb buds and eyes, organs which undergo extensive differentiation and morphogenesis during the stages studied here. It therefore follows from our observations that maximal induction of p53 SST activity is seen preferentially in faster-dividing, less-differentiated elements.

In conclusion, the ability of endogenous p53 to be activated as a transcription factor appears to be inversely correlated to the stage of maturation of the embryo and the extent of differentiation of the particular tissue.

Maximal promoter activation requires both wt p53 alleles: evidence for haploinsufficiency in vivo

The strong radiation inducibility of the transgenic *mdm2* promoter indicated that its activity is p53 dependent in this system. We wished to confirm formally the p53 dependence of this induction, and to assess the p53 dependence of the *lacZ* expression observed in the uninduced state. Therefore, *mdm2* P2–*lacZ* transgenic mice were mated to p53+/– mice. A resultant *lacZ* transgenic male, heterozygous for the p53 null allele, was mated to a p53+/– female not carrying the *lacZ* transgene. The pregnant female was irradiated at day 10.5 p.c. and sacrificed 3 h later. Individual embryos from the same litter were isolated separately. The yolk sac of each embryo

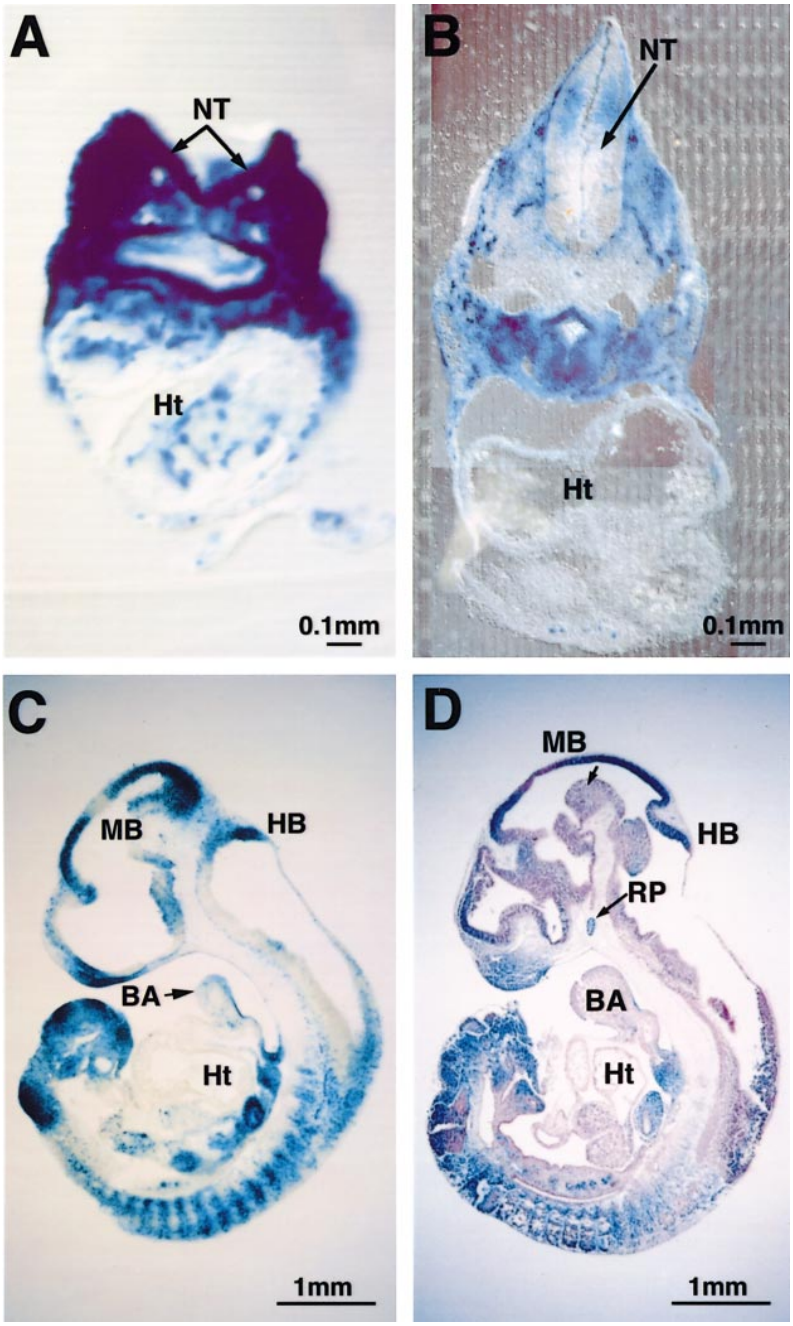

Fig. 3. Histological analysis of *lacZ* staining in irradiated embryos. Embryos were isolated 3 h after exposure of the pregnant female to 5 Gy of γ radiation, and then subjected to whole mount staining with X-Gal followed by further analysis by different sectioning procedures. (**A**) Vibratome cross-section of an E8.5 embryo. (**B**) Vibratome cross-section of an E10.5 embryo. (**C**) Vibratome sagittal section of an E10.5 embryo. (**D**) Eosin-stained sagittal paraffin section of an E10.5 embryo. Abbreviations: BA, branchial arches; HB, hindbrain; Ht, heart; MB, midbrain; NT, neural tube; RP, Rathke's pouch.

was used for DNA extraction and PCR analysis. The embryos were stained for *lacZ*. In parallel, the p53 and *lacZ* genotype of each embryo was determined by PCR analysis using two primer pairs which distinguish between the wt p53 allele and the knock-out p53 allele, and a third pair of primers specific for the *lacZ* transgene. Representative p53+/+, p53+/– and p53–/– embryos are displayed in Figure 4A–C); the corresponding PCR patterns are shown in Figure 4D.

Our results confirm that the strong induction of *lacZ* activity by IR is indeed p53 dependent, as it does not occur in p53 null mutant embryos (compare Figure 4A and C). On the other hand, the pattern of *lacZ* expression in p53 null mutant embryos (Figure 4C) was practically indistinguishable from that seen in unirradiated p53+/+ embryos of the same age (compare with Figure 2C). The basal pattern of expression, seen in the absence of DNA damage, therefore appears to be p53 independent. Whether this basal pattern represents physiological sites of p53-independent activity of the *mdm2* P2 promoter is presently unclear.

Importantly, Figure 4 reveals a striking dependence of

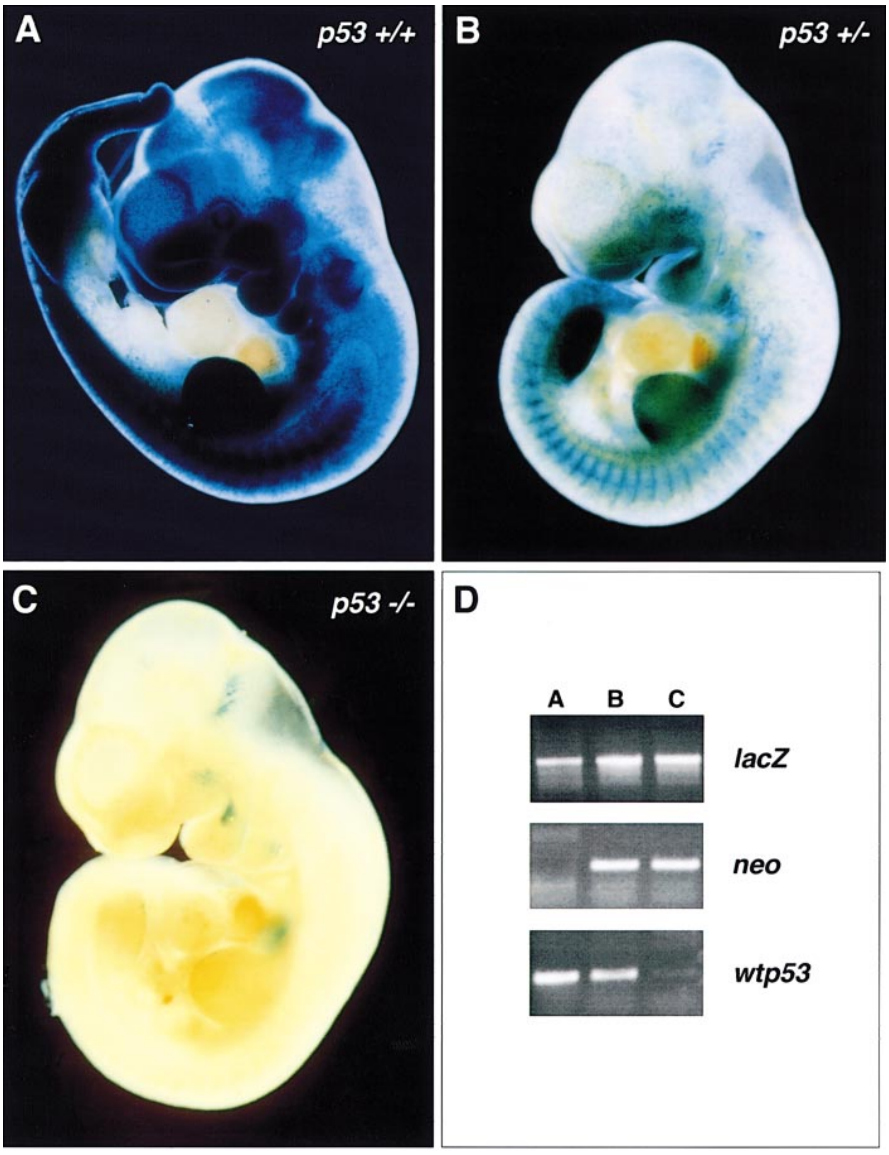

Fig. 4. Induction of *lacZ* activity by radiation in transgenic E10.5 embryos of different p53 genotypes. A p53+/– female was mated to a male heterozygous for the p53 null allele and for the *mdm2* P2–*lacZ* transgene. Embryos of a single litter (a total of 11 embryos) were isolated 3 h after γ irradiation and stained for *lacZ* activity. Incubation with the X-Gal substrate was for 16 h. In parallel, DNA was extracted from the yolk sac of each embryo and subjected to PCR analysis in order to monitor for the presence of the *lacZ* transgene, as well as the wt and the null (neo^r) p53 alleles. One representative *lacZ*-positive embryo of each p53 genotype is shown (**A–C**); the p53 genotype is indicated on the upper right side of each panel. The corresponding PCR products for each of the three embryos are shown in (**D**). The weak wt p53 band in the reaction with embryo C DNA is probably due to a minor contamination of the yolk sac sample with cells of maternal origin.

lacZ induction on wt p53 gene dosage. While p53-dependent activation of the reporter is clearly seen in irradiated p53+/– embryos (compare Figure 4B and C), the intensity of this staining is far below that obtained in p53+/+ embryos (compare Figure 4A and B). In fact, the picture shown underestimates the true difference between the homozygous and heterozygous wt p53 genotypes: in order to maximize staining intensity in p53–/– and p53+/– embryos, incubation of all embryos with the *lacZ* substrate was carried out for 16 h. Consequently, the staining in Figure 4A has already reached saturation. Conspicuous staining was already seen in the p53+/+ embryos after 4 h of incubation (see Figure 2D), while staining in p53–/– and p53+/– littermates was still very faint at that time (data not shown). Hence, the actual difference in the extent of *lacZ* expression between embryos containing one wt p53 allele and those containing two such alleles is even greater than implied by Figure 4. These profound differences between p53+/+ and p53+/– embryos were consistent within the same litter, as well as across litters (data not shown).

The data also suggest that the threshold for activation of the p53 SST response by DNA damage varies among individual tissues. Consequently, the presence of a single wt p53 allele is sufficient for at least partial induction of the transgenic *mdm2* promoter in some cell types, whereas in others no induction can take place unless both wt p53 alleles are retained. For instance, while the hind limbs, visceral arches and ventral parts of the olfactory bulbs display a substantial p53 response to IR even in p53+/–

embryos, the hindbrain and midbrain show only minimal induction, unlike the prominent activation visible at these sites in p53+/+ littermates.

Taken together, our findings argue strongly that, at least for the *mdm2* P2 promoter, a full complement of wt p53 genes is required for optimal SST response to DNA damage. Retention of a single wt p53 allele results in an incomplete response. The extent of haploinsufficiency varies from tissue to tissue, being partial in some tissues and more severe in others. Thus, relatively moderate differences in basal levels of p53 can already dictate whether or not a particular cell, when exposed to a stress signal, will accumulate enough p53 to drive the efficient activation of downstream target genes.

Discussion

We describe here the generation of a transgenic mouse model for studying the biochemical activation of p53 *in vivo* in response to genotoxic stress. The mice carry a reporter gene, β-galactosidase (*lacZ*), under control of a physiologically relevant p53-dependent promoter derived from the *mdm2* gene (*mdm2* P2). Induction of *lacZ* expression monitors the extent of SST by p53—considered the main, though not only, biochemical function through which p53 mediates its various biological effects (Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996). The present analysis is confined to the induction of p53 SST in the developing mouse embryo, following exposure to IR. Two main conclusions can be drawn from this study. First, the potential for induction of p53 SST by DNA damage is not intrinsic to every cell in the embryo; rather, it is spatially and temporally restricted. The spectrum of sites which exhibit detectable activation after exposure to IR varies with developmental age, and also between different compartments of the developing embryo and between different zones of the same compartment. In general, maximal inducibility of p53 SST correlates with a less differentiated, more highly proliferative state. This is perhaps illustrated most vividly by the gradient of induction seen along the anterior–posterior axis at E10.5 (Figure 2D).

At E10.5, all cells of the embryo express high levels of p53 mRNA (Schmid *et al.*, 1991). Hence regulation of p53 function, at least in response to DNA damage, is not at the transcriptional level. Presumably p53 mRNA is translated in all tissues but the protein is degraded rapidly and thus does not accumulate to appreciable levels. However, these labile p53 molecules may serve as a constantly available pool for the efficient accumulation of functional protein, once they become stabilized in response to appropriate stress signals. In addition, the constitutive reservoir of p53 mRNA may give rise to more protein through DNA damage-induced relief of inhibitory translational control (Mosner *et al*., 1995). Our data imply that tissue- and cell type-specific factors can determine whether or not this potential for rapid overproduction and accumulation of functional wt p53 is actually materialized. At later stages of development, further restrictions may be imposed on the p53 response by limiting p53 mRNA expression to only a subset of cells and tissue types (Rogel *et al*., 1985; Schmid *et al.*, 1991).

There is generally a good correlation between extensive accumulation of p53 after DNA damage and the likelihood of apoptosis (Komarova *et al.*, 1997, accompanying paper; D.MacCallum and P.Hall, personal communication). Induction of p53-mediated apoptosis may well require higher levels of p53 protein than for other biological activities of p53. Consequently, even quantitative differences in levels of DNA damage-induced p53 activation may suffice to dictate which cells will undergo apoptosis and which will fail to do so. Moreover, the distinction between apoptosis and viable growth arrest may be a function of the extent of DNA damage (Gottlieb *et al.*, 1996). Even though this distinction is not necessarily always dependent on SST (Caelles *et al.*, 1994; Haupt *et al.*, 1995), it does suggest that there are defined thresholds of DNA damage below which some p53 effects do not take place. It is quite conceivable that higher doses of IR than employed by us might have resulted in induction of SST, as well as of apoptosis, at sites where no prominent induction is seen at 5 Gy of IR.

In the adult mouse, tissue-specific restriction of the p53 response is already well documented (Clarke *et al.*, 1994; Merritt *et al.*, 1994; Midgley *et al.*, 1995). This is manifested in the limited spectrum of cells which accumulate p53 protein in response to DNA damage, and further in the fact that only a subset of the latter go on to p53-dependent apoptosis; recent studies (Komarova *et al.*, accompanying paper; D.MacCallum and P.Hall, personal communication) extend this picture also to activation of p53 SST. In the adult mouse, as in the developing embryo, cells which exhibit the strongest p53 response generally belong to the highly proliferative, relatively undifferentiated compartment. The increased sensitivity of such cells to DNA damage-mediated activation of p53 is consistent with the notion that a key role of p53 is to prevent the propagation of genomic aberrations. Such aberrations are likely to be most deleterious to the organism when occurring in cells with a high proliferative potential, which can accumulate further alterations during subsequent rounds of replication and are more likely to give rise to malignant progeny. It would therefore be advantageous for the organism to maintain in such cells a high potential for activation of the p53 pathway. This conjecture is in line with the remarkable susceptibility of p53 null mice to radiation-induced tumorigenesis (Kemp *et al*., 1994), as well as their propensity to develop tumors which are associated with chromosomal instability (Donehower *et al.*, 1995). It is also consistent with the observation that the absence of functional wt p53 promotes teratogenesis in the mouse (Nicol *et al.*, 1995; Norimura *et al.*, 1996). Thus, induction of p53-dependent apoptosis in embryonic tissues exposed to mild genotoxic stress prevents the accumulation and propagation of cells with defective DNA. In this way, p53 safeguards the developing embryo against teratogenic defects. It should be noted, however, that exposure of the embryo to more severe genotoxic stress actually promotes teratogenesis (Wubah *et al.*, 1996). This effect, most dramatic in particularly sensitive organs like the developing eye, is caused by excessive p53-dependent apoptosis that cannot be compensated by the proliferation of unaffected cells.

In our study, embryos were exposed to 5 Gy of IR. This dose is highly lethal for normal mouse embryos; all embryos exposed to this dose at E10.5 died *in utero* (data

not shown). In fact, even exposure to only 2 Gy is sufficient to induce 60% prenatal deaths in p53+/+ embryos (Norimura *et al.*, 1996). On the other hand, p53–/– embryos exposed to 2 Gy display increased survival, accompanied by excessive teratogenic defects (Norimura *et al.*, 1996). Here, too, p53+/– embryos exhibit a severely compromised phenotype, indicative of haploinsufficiency with regard to the anti-teratogenic effect of wt p53. The most frequent developmental defects induced by IR in the absence of p53, as well as in p53+/– embryos, affect the limbs and tail (Norimura *et al.*, 1996). Most remarkably, these are also sites of maximal induction of p53 SST activity, as revealed in the present study (e.g. Figure 2D). These observations therefore support the notion that the anti-teratogenic effect of p53 is tightly coupled with its ability to become biochemically activated, in a tissue-specific manner, in response to genomic damage.

The second main conclusion from the present study has to do with the marked degree of haploinsufficiency revealed in the p53+/– embryos. At least as judged from the extent of activation of the *mdm2* P2 promoter, loss of one wt p53 allele severely compromises the p53 SST response. In other words, in many tissues the presence of only one p53 allele is already sufficient to prevent the cell from mounting a full p53 response when required to do so. Such reflection of p53 gene dosage is expected to result in less efficient biological effects, especially in situations where high amounts of active p53 are required. Such a dosage effect has indeed been described for radiation-induced, p53-mediated apoptosis in the mouse thymus (Clarke *et al.*, 1993; Lowe *et al.*, 1993) and intestinal crypt (Clarke *et al.*, 1994).

It has been noted early on that mice heterozygous for p53 are more prone to cancer than p53+/+ mice (Donehower *et al.*, 1992; Kemp *et al.*, 1993; Jacks *et al.*, 1994), a situation reminiscent of the human Li–Fraumeni syndrome (Malkin *et al.*, 1990). The easiest way to account for this difference in cancer predisposition is by proposing a purely statistical explanation. Hence, the likelihood to lose p53 function completely is much greater on a p53+/– background than on a p53+/+ one, because all it takes in p53+\– individuals is the loss of the single remaining wt p53 allele or its mutational inactivation. This statistical explanation is largely correct, as indicated by the frequent loss of the wt p53 allele in tumors arising in mice heterozygous for p53, as well as in Li–Fraumeni patients. However, further analysis has now given rise to the surprising observation that a significant fraction of tumors in p53+/– mice do appear to retain the wt p53 allele, and even in a functionally competent state (L.Donehower, personal communication). p53 haploinsufficiency may therefore contribute directly to tumorigenesis, by confining p53 activity in particular cells to levels which are below the threshold required for the effective inactivation or elimination of these cells once they become aberrant.

The extent of observed haploinsufficiency is likely to depend largely on the particular activity of p53 which is being evaluated. Specifically, in the system described here, full activation of the *lacZ* reporter probably requires the binding of two p53 tetramers to the two adjacent p53 response elements (p53RE) residing within the *mdm2* P2 promoter (see Figure 1). In fact, promoters containing only a single *mdm2*-derived p53RE are activated by p53 far less efficiently than the full P2 promoter, when assayed in transfected cells in culture (T.Juven-Gershon and M.Oren, unpublished observations). Moreover, the presence of two rather than one p53REs, sometimes positioned at a rather large distance from each other, is emerging as a feature common to many p53 target genes, including not only *mdm2* but also p21 (El-Deiry *et al.*, 1995) and cyclin G (Zauberman *et al.*, 1995). Such a feature may be essential for optimal activation of these genes by p53 (Stenger *et al.*, 1994). A 2-fold reduction in p53 protein levels may, therefore, cause a much larger than 2-fold decrease in the proportion of target promoters that can still bind two p53 tetramers each, especially if cellular p53 concentrations are already limiting beforehand. Our data suggest that the latter may indeed be true. Moreover, the degree to which p53 concentration is already limiting in the p53+/+ state appears to vary among different cell types; consequently, the severity of haploinsufficiency in p53+/– embryos also exhibits tissue-specific variations. Finally, it is conceivable that biochemical activities of p53 other than SST, if not requiring tetramerization, will be less sensitive to p53 gene dosage. Processes dependent on such activities may thus still take place normally in p53 heterozygous animals, as well as in cells of Li–Fraumeni individuals.

Materials and methods

Mouse strains

CB6/F1 female mice were used as fertilized oocyte donors. Pseudopregnant CD-1 outbred albino females were used as foster mothers for embryo transfer. Mice carrying one or two p53 null alleles on a 75% C57BL/6, 25% 129/Sv genetic background (Donehower *et al.*, 1992) were kindly provided by L.Donehower through V.Rotter.

Generation of transgenic mice

The *Apa*I–*Nsi*I fragment of the murine *mdm2* gene (Juven *et al.*, 1993), containing the p53-dependent P2 promoter (Barak *et al.*, 1994), was inserted into plasmid pSL301, and then excised and religated between the *Xba*I and *Sal*I sites of the *lacZ*-containing plasmid pPD46.21 (Goldhamer *et al.*, 1995). The resultant plasmid was denoted pPD/mdm2P2. The *Pst*I–*Not*I fragment of pPD/mdm2P2 was purified and used for microinjection. This fragment contains the *mdm2* P2 promoter followed by the *lacZ* gene and the SV40 early polyadenylation site, and is practically devoid of vector plasmid sequences; the encoded *lacZ* protein also includes the SV40 large T antigen nuclear localization signal.

Fertilized oocytes were isolated from CB6/F1 females, microinjected with the above *Pst*I–*Not*I fragment, and maintained overnight at 37°C in M16 medium (Sigma). Embryos that had developed into the two-cell stage were transferred into CD-1 recipients. Transgenic founders were identified by extraction of DNA from tails and subsequent Southern blot analysis (Hogan *et al.*, 1994), with the microinjected DNA fragment serving as a probe. Each founder was analyzed for transmission of radiation-inducible *lacZ* expression by mating with non-transgenic females, exposure of the females to 5 Gy of γ radiation from a Co^{60} source at day 10.5 p.c., isolation of the embryos 3 h later and staining for *lacZ* activity using X-Gal (5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MBI Fermentas) as substrate.

Embryo analysis

Embryos were isolated at different stages of development (8.5, 10.5 and 12.5 days p.c.) and subjected to whole mount X-Gal staining (Hogan *et al.*, 1994). Briefly, embryos were fixed for 15–45 min in a solution containing 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in phosphate-buffered saline (PBS; pH 7.4). Fixed embryos were washed twice in PBS, and subjected to staining for 4–16 h in a solution containing 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS.

For genotyping, the yolk sac was isolated separately from each embryo, and DNA was extracted in 50 mM Tris–HCl pH 8.0, 100 mM

EDTA, 0.5% SDS and 0.5 mg/ml proteinase K for 6 h at 55°C, followed by phenol extraction and ethanol precipitation. The DNA was subjected to PCR analysis, using three different sets of primers specific for the *mdm2* P2–*lacZ* transgene, the disrupted (*neo*-containing) p53 allele and the wt p53 allele, respectively. The p53 allele-specific primers were as described in Timme and Thompson (1994). The *mdm2* P2–*lacZ* primers were: 5′-GCATTTGAGAGCTATTGCCG-3′ (*mdm2* primer) and 5′-GATTCATTCCCCAGCGACC-3′ (*lacZ* primer).

Histological analysis

Embryos that had already been fixed and stained for *lacZ* as above were fixed again by incubation in 4% paraformaldehyde (in PBS) for 2–8 h, and then processed further for paraffin sections or vibratome sections, as described below.

Paraffin sections. Fixed embryos were dehydrated in ethanol, isopropanol and toluene according to standard procedures (Hogan *et al.*, 1994), embedded in Paraplast Plus (Monoject Scientific), and microtome sections were prepared at a thickness of 10 μm. Sections were rehydrated and stained in 0.1% eosin.

Vibratome sections. Fixed embryos were embedded in a gelatin/albumin block by placing them in 4 ml of an aqueous solution containing 0.4% gelatin, 25% albumin and 15% sucrose, and then adding 0.4 ml of 25% glutaraldehyde. Blocks were subjected to vibratome sectioning at a thickness of 20–30 μm.

Photographs were taken using bright field or differential interference contrast (Nomarski) microscopy.

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