Transcriptional regulation of MDR genes

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Abstract

The emergence of resistance in a tumor population is most often associated with a dysregulation of gene expression, usually at the level of transcription. A major goal in the field of cancer chemotherapy is to define the mechanisms underlying transcriptional regulation of drug resistance genes in an effort to identify targets for therapeutic intervention. Recently, considerable progress has been made in identifying the molecular mechanisms involved in the transcriptional regulation of the P-glycoprotein (Pgp) gene. When overexpressed in tumor cells, Pgp confers resistance to a variety of chemotherapeutic agents; this resistance has been termed MDR (multidrug resistance). Moreover, Pgp is a normal component of a variety of highly differentiated cell types and, as such, is regulated by both internal and external environmental stimuli. In this review, we will discuss the current knowledge regarding the DNA elements and protein factors involved in both constitutive and inducible regulation of Pgp transcription in normal and tumor cells.

Introduction

Regulation at the level of RNA transcription plays a critical role in determining a cell's response to a variety of stimuli, both internal and external. Some gene products are required for the survival of all cells types; hence, the expression of their genes is often protected from the effects of environmental fluctuations. The regulation of other genes is subject to continual flux as a consequence of changes in their cellular milieu. The P-glycoprotein (Pgp) genes fall into this latter category.

P-glycoproteins are part of a gene family that has been highly conserved throughout evolution, from yeast to humans. Most of the studies of Pgp function and regulation have focused on the gene families in human and rodents (reviewed in Schustik, 1995). In general, Pgp genes are divided into three classes, based on homology and role in the MDR phenotype. Classes I and II include Pgp homologs whose overexpression is associated with the MDR phenotype; Class III genes do not appear to play a role in drug resistance. While rodent cells include genes in all three

classes, human cells lack a Class II homolog. Therefore MDR1 is the only human gene involved in drug resistance.

P-glycoprotein was first identified as a consequence of its overexpression in multidrug resistant cells (Juliano, 1976). Initial studies of MDR cell lines indicated that this overexpression was the result of gene amplification, a mechanism that had been identified as the mediator of overexpression of other genes, such as dihydrofolate reductase (DHFR), in drug-resistant cells (reviewed in Banerjee, 1995). However, subsequent studies in other cell lines indicated that increased levels of P-glycoprotein and its cognate mRNA could occur in the absence of a commensurate increase in gene copy number (Scotto, 1986), thus implicating a transcriptional mechanism in the activation of P-glycoprotein genes in drug-resistant cells. Failure to identify gene amplification as a mechanism of overexpression of Pgp in clinical samples further supports the notion that transcriptional regulation may mediate the overexpression of Pgp in human tumors.

In addition to their activation during the acquisition of the MDR phenotype, Pgp genes are differentially

expressed in normal tissues, both as a consequence of differentiation triggers and in response to environmental challenges (reviewed in Fardel, 1996; Borst, 1997; Ling, 1997). In general, a single Pgp isoform predominates in specialized cells within a given tissue. For example, the high levels of human Class I MDR1 expression are found in the adrenal gland, kidney, jejunum, colon and the endothelial cells of the blood-brain and blood-testes barrier, while human class III gene expression predominates in the liver. In addition to cell- and tissue-specific expression, environmental factors such as heat shock, cytokines, hormones, differentiation agents, chemotherapeutics, UV- and X-irradiation, receptor agonists (Furuya, 1997), oncogenes and tumor suppressor genes can all influence the levels of specific Pgp isoforms in different systems. Therefore, Pgp gene expression is controlled by a highly complex interplay of a variety of factors that are involved in multiple regulatory pathways.

Transcription of P-glycoprotein Genes

Transcriptional regulation of eukaryotic protein-coding genes is extraordinarily complex, relying on the cooperation of multiple protein and nucleic acid factors to achieve the specificity of response necessary for cell survival. Considerable effort has been directed at identifying and characterizing these transcriptional catalysts, and understanding how the modulation of various components of the cell's transcriptional machinery can influence the fate of the cell and ultimately, the organism. In general, these determinants can be divided into three classes: those that direct initiation of basal transcription, those that are involved in the activation or repression of basal expression, and those that are involved in regulation by chromatin. This review will address the role of all three transcription factor classes in the regulation of Pgp genes, with a primary focus on the transcription of the human Class I homolog, MDR1.

Transcription initiation in Pgp genes

Transcription initiation is defined as a series of events leading to the formation of the first phosphodiester bond in the nascent RNA transcript. Early in the studies of transcription by RNA polymerase II (Pol II), the enzyme responsible for the synthesis of protein-coding RNAs, it became clear that the polymerase alone was not sufficient for specific initiation from a

DNA template. During the past decade, a tremendous effort has been directed at the purification and characterization of the additional protein factors required for basal transcription, which are referred to as GTFs (general transcription factors, reviewed in Orphanides, 1996; Hampsey, 1998).

TATA-containing promoters

The first genes to be studied were those that contained a TATA box, a DNA element found ~30 bp upstream of the transcription start site that is involved in the positioning of the polymerase complex. Recognition of the TATA box by TFIID, a multisubunit protein, is the first event in the formation of the Pol II-GTF complex on TATA-containing promoters. It appears that the mouse Class I and Class II Pgp promoters, as well as the rat Class II promoter, fall into this category, since they all contain TATA elements upstream of their transcription start sites (Figure 1). However, the function of these elements is inferred from their sequence, since their role in transcriptional initiation of these Pgp promoters has not yet been tested.

Single start site TATA-less promoters

A second class of Pol II promoters includes those that lack a TATA element and initiate transcription at a single start site. Many of these TATA-less promoters contain a second basal element, referred to as the initiator (Inr), which encompasses the transcription start site and is sufficient to position the basal machinery in the absence of a TATA motif (reviewed in Smale, 1997). By random mutagenesis, computational analysis and functional assays, a consensus initiator sequence, PyPyA(+1)NT/APyPy, was identified (Javahery, 1994). The human MDR1 promoter contains such a consensus sequence.

Transcription of the MDR1 gene begins at two major sites (+1 and +5) which lie 140 and 136 bp upstream of the translation start site, respectively (Ueda, 1987; Cornwell, 1990; Madden, 1993). An element extending from -13 to +23, which contains a consensus Inr sequence, has been shown to be required for accurate initiation of transcription from this promoter. The first indication of the presence of an Inr element in the MDR1 promoter came from in vitro studies, which showed that sequences from -132 to +82 were sufficient for accurate initiation, while deletion of sequences downstream of +5 decreased correctly initiated transcription to undetectable levels (Cornwell, 1990). Subsequently, transient transfection studies indicated that sequences between -6 and +11

were sufficient for proper initiation of transcription in vivo (van Groenigen, 1993; Madden, 1993). Taken together, these studies confirm that the MDR1 initiator is functional both in vivo and in vitro, and that sequences immediately downstream of the +1 site are required for accurate and efficient transcription.

In some MDR cell lines and human tumors, MDR1 transcripts initiating upstream of +1 have been identified. Initially believed to arise from initiation at an upstream MDR1 promoter, a recent study of these alternative transcripts in one MDR cell line and several tumors suggests that they may actually be transcribed from a heterologous promoter that has translocated to a site 5' of the MDR gene (Mickley, 1997; see below).

Multiple start site TATA-less promoters

Although many TATA-less promoters can initiate transcription at a narrow cluster of start sites surrounding the major +1 site, many other TATA-less promoters initiate transcription within a broad window encompassing ~ 100 bp. The prototype of this third class of Pol II promoters is found in the hamster class I Pgp gene (pgp1). In hamster, pgp1 transcription usually begins at a site 67 bp upstream of the homologous +1 site in the MDR1 promoter (Figure 1). However, in some Chinese hamster cell lines the development of resistance to MDR drugs is accompanied by the utilization of additional start sites 32, 42, 52 and 67 bp downstream of the +1 site utilized in drug sensitive cells (Ince, 1995a). A downstream element, MED-1, which is conserved in multiple start site TATA-less promoters, is required for the activation of pgp1 transcription in MDR cells (Ince, 1995b).

Activation and repression of transcription of Pgp genes

The basal transcription complex, containing Pol II and the GTFs, is sufficient to initiate transcription of protein-coding genes. However, the efficiency with which these genes are expressed is determined by other factors, collectively referred to as activators and repressors. These factors function through a direct interaction with DNA elements within the promoter and/or via protein-protein interactions with other transcription factors or the basal machinery. A myriad of DNA binding elements is located within a promoter. Whether a gene is activated or repressed in a given cell type or under different physiological conditions is determined by the presence, complexity and accessibility of DNA response elements within the promoter, as

well as by the complement of transcription factors that interact with these elements (reviewed in Latchman, 1997).

With few exceptions, the majority of transcription factors implicated in the regulation of Pgp genes exert a positive effect on their transcription. Some regulators appear to be operative in most cell types under standard growth conditions (constitutive activators), while others function upon induction by external factors (inducible activators). However, most DNA elements are involved in both constitutive and inducible transcription, and in the majority of cases the complement of binding proteins involved in these diverse activities is not fully understood. For ease of presentation, the factors and DNA elements known to be involved in transcription of P-glycoprotein genes will be broadly classified as 'constitutive' and 'inducible', where 'constitutive' refers to the transcription of Pgp genes in a given cell lines assayed under standard culture conditions, and 'inducible' refers to the regulation of this transcription by exogenously added factors or treatments. It must be kept in mind, however, that none of these transcriptional components operate in a vacuum, and that the complex interplay of multiple factors determine transcriptional output.

Constitutive regulators

The SP1 site Sp1 is a ubiquitous transcription factor and its binding site is almost invariably found in promoters lacking a TATA element (reviewed in Lania, 1997). The human MDR1 promoter is no exception. DNase I footprint analysis of the MDR1 promoter revealed a protected region on both strands extending from -61 to -43 relative to the major start site of transcription. A GC-box (GGGGCGTGGGCTGA) was located within this region (-56 to -43) and was found, by gel shift and antibody analyses, to contain an SP1 binding site (Cornwell, 1993a; Sundseth, 1997). Mutations between -61 and -43 resulted in a 6-fold decrease in promoter activity when the mutant MDR1 promoter construct was transiently transfected into KB-8-5 cells, and SP1 was shown to activate the MDR1 promoter when co-transfected into *Drosophila* cells (Cornwell, 1993a). In addition, the activity of the SP1 phosphoprotein is regulated by protein kinase A, and it has been suggested that the down-regulation of MDR1 by the PKA inhibitor 8-Cl-cAMP (Scala, 1995; Rohlf, 1995) is mediated by SP1 (Rohlf, 1998). Although initially proposed to be a critical factor for the basal transcription of MDR1 in all cells, recent

evidence suggests that this requirement may be cell-specific, since mutation of this element in several human neuroblastoma cell lines had only a small effect on basal transcription (Thayer, S. and Scotto, K., unpublished).

The TATA-less rat *mdr2* gene is also regulated by Sp1 (Brown, 1996). However, in contrast to its location directly upstream of the initiation site in the MDR1 promoter, the SP1 site in the *mdr2* promoter is located downstream of what appears to be multiple transcription start sites. This region was shown to bind Sp1 *in vitro*, and overexpression of Sp1 was shown to activate *mdr2* transcription through this site in *Drosophila* transfection studies.

NF-R1 and NF-R2 Two additional transcription factors that interact with GC-rich regions in the MDR-1 promoter have been purified from K562/ADM cells and designated NF-R1 and NF-R2. NF-R1, a 110 kD protein, interacts with sequences between -123 and -115 and between -56 and -45 (the Sp1/EGR1 site) (Ogura, 1992). A series of 2 bp scanning mutations within either the upstream or the downstream GC-box resulted in a 2–3 fold increase in promoter activity, suggesting the presence of a repressor binding site. While a repressor site had already been described in the region of -110 (Cornwell, 1990), the presence of a repressor in the downstream site appears to be in conflict with the aforementioned study in which SP-1 and EGR-1 functioned as activators at the downstream GC-box. However, different mutations and different cell lines were analyzed in the two studies, preventing direct comparison of the results. NF-R2 is a 75 kD protein which interacts with sequences between -119 and -111 (Takatori, 1992). Because this overlaps the binding site for NF-R1, and mutations in the site reduced the binding of both complexes, the relative role of NF-R1 and NF-R2 in MDR1 regulation through the upstream GC-box is not yet clear.

The inverted CCAAT box (Y-box) Another element that has been shown to be involved in the basal expression of the human MDR1 gene in many cell types is the inverted CCAAT box (also referred to as the Y-box) located at position -82 to -73 within the promoter (Goldsmith, 1993). Deletion of this element between -85 and -70, as well as point mutations at -78 and -75, caused a 5–10 fold decrease in promoter activity in cell lines derived from human colon, ovarian carcinoma, liver, and osteosarcoma cells. Multiple transcription factors are known to interact with inverted CCAAT

elements; both NF-Y (Sundseth, 1997; Jin, 1998) and YB-1 (Bargou, 1997; Ohga, 1996; Ohga, 1998) have been implicated in MDR1 regulation through this element. In fact, a recent study of MDR1 transcription in the human breast cancer cell line, MCF7, relates the nuclear localization of YB-1 to the activation of MDR1 (Bargou, 1997). In drug-sensitive MCF-7 cells, YB-1 was localized in the cytoplasm, yet in a multidrug-resistant MCF-7 subline which expressed high levels of MDR1, YB-1 was found in the nucleus, consistent with a role in MDR1 activation. Nuclear-localized YB-1 was also observed in intrinsically resistant human tumors in which high levels of Pgp were present. Taken together, these data suggest that YB-1 plays a role in MDR1 transcription. Moreover, activation of MDR1 by environmental stress is also mediated by the inverted CCAAT box, and both NF-Y (Hu, Z. and Scotto, K., unpublished) and YB-1 (Ohga, 1996) have been implicated in this process. Finally, induction of MDR1 transcription by the differentiation agent, sodium butyrate, requires an intact CCAAT box and NF-Y (Jin, 1998). The role of the CCAAT box and its cognate binding factors in transcriptional induction of MDR1 by these agents will be discussed in more detail in later sections.

Using DNase I footprinting analysis, an inverted CCAAT element was also identified between -70 and -61 in the mouse *mdr1b* promoter (Yu, 1995). This element was found to bind NF-Y. Transient transfection studies showed that the inverted CCAAT box was critical for the constitutive expression of an *mdr1b* reporter construct in mouse adrenal Y-1 cells, since mutation of this site decreased transcription at least five-fold. Interestingly, these mutations also effected activation of the promoter by the CAAT Enhancer binding Protein, C/EBP β , which interacts at a different site, suggesting that NF-Y and C/EBP β cooperate to regulate the *mdr1b* promoter.

The AP-1 site There is indirect evidence that the AP-1 complex may be involved in P-glycoprotein gene transcription. AP-1 is the general term for transcription factor complexes composed of members of the Fos and Jun oncogene families. They are constitutively expressed in many cell types and DNA-binding by the complex is induced by serum stimulation, phorbol esters and a variety of growth factors. They are also induced by various stress stimuli such as UV irradiation (reviewed in Karin, 1997).

Elevated levels of c-Fos have been shown in a number of drug-resistant cell lines when compared

to their drug-sensitive counterparts (Bhushan, 1994; Kim, 1994). Chinese hamster ovary cells that have a mutant form of PKA, a kinase that is known to activate AP-1, have decreased expression of P-glycoprotein (Cvijic, 1997) and a PKA inhibitor, H-87, was found to decrease expression of human MDR1 in the P388 leukemia cell line (Kim, 1993). However, PKA has also been implicated in regulation by SP1 (Rohlf, 1998), complicating the interpretation of these data. A similar line of circumstantial evidence comes from studies of the c-Jun NH2-terminal protein kinase (JNK), which also activates AP-1. In human KB-3 cells adriamycin, vinblastine and etoposide (VP-16) activated JNK, and this activation was associated with an increase in MDR1 expression at the mRNA level (Osborne, 1996). Two multidrug-resistant variants of KB-3, KB-A1 and KB-V1, both showed increased basal levels of JNK activity over the KB-3 parental cell-line.

Putative AP-1 binding sites have been reported in human and rodent class I P-glycoprotein promoters. However, although the AP-1 elements in the promoters of the hamster (–55 to –49) and mouse (–117 to –123) are required for efficient transcription of those genes (activation in hamster (Teeter, 1991a) and repression in mouse (Ikeguchi, 1991)), there is no apparent role for AP-1 in the basal activity of the rat promoter, and the human promoter has not yet been functionally evaluated. Moreover, there is no direct evidence that fos and jun proteins are involved in complex formation on Pgp promoters. Therefore, despite considerable circumstantial evidence, there is no conclusive proof that AP-1 is actually involved in the transcriptional regulation of P-glycoprotein genes. Further studies in this area are warranted.

Tissue-specific activators Although not considered constitutive in the sense that they are involved in transcription in all cells, tissue-specific activators are generally involved in constitutive expression in the cells in which they are expressed and will therefore be discussed in this section. However, other factors which are activated in specific cells/tissues in response to external challenges will be discussed elsewhere.

The normal expression of Pgp isoforms is tissue-specific in humans and rodents. Differential regulation of Pgp transcription has been studied most extensively in mouse and rat liver, using hepatoma cell lines, primary hepatocyte cultures and regenerating liver as models (Marino, 1990; Nakatsukasa, 1993; Zhao, 1993; Lee, 1994; Schuetz, 1995; Song, 1995). In tran-

sient transfection experiments, a liver-specific element was identified between –181 and –164 in the mouse Class II *mdr1b* promoter (Song, 1995). In vitro DNase I footprinting and gel mobility shift assays identified a protein binding to this region in both hepatoma and non-hepatoma cells. However, mutation of the liver-specific element in an *mdr1b*-CAT construct only reduced activity in mouse hepatoma cell lines (Bprc1, Hepa1c1c and Hepa1-6) and not in a non-hepatoma cell line (NIH3T3) or in a human epidermal carcinoma cell line (HeLa). Moreover, when cloned upstream of a thymidine kinase promoter in either orientation, the *mdr1b* promoter region between –185 and –155 activated this heterologous construct in a hepatoma-specific manner, confirming its role as a cell-specific enhancer. A homologous element was identified in the rat *mdr1b* (*pgpa*) gene (Zhao, 1996).

In rat, *pgp2* is the least abundant isoform in liver, yet it is overexpressed in primary rat hepatocyte cultures. However, it was found that this overexpression resulted from the specific stabilization of the *pgp2* mRNA and it did not involve activation at the transcriptional level (Schuetz, 1995). Induction of Class II and III Pgp genes was also observed during rat liver regeneration. Initial studies suggest that this activation may also involve a post-transcriptional mechanism.

It should be noted that a putative tissue-specific MDR enhancer located approximately 10 kb upstream of the MDR1 promoter had been identified (Kohn, 1990). However, subsequent studies showed that this element was located on a different chromosome (20 q13.1) than MDR1 (chromosome 7), suggesting that a chimeric genomic clone which included heterologous sequences was isolated in the initial study (Germann, 1994). Thus, it is unlikely that this element regulates the expression of the MDR1 gene *in vivo*.

Oncogenes and tumor suppressor genes High levels of MDR1 expression are sometimes observed in the absence of previous exposure to chemotherapeutics, even in tumors that originate from normal tissues which have little or no expression of MDR1. Hence, constitutive MDR1 gene expression may be influenced in some cells by components that are involved in malignant transformation. Mutations in *ras* and *p53* proteins have been detected frequently in many different cancers which also express P-glycoprotein, and several studies suggest that these proteins play a role in P-glycoprotein expression.

p53 A number of studies have addressed the effect of p53 on Pgp transcription, producing a myriad of seemingly contradictory results. Initial studies in murine NIH3T3 cells showed that the activity of a human MDR1-CAT reporter (Chin, 1992) and a hamster pgp1-CAT reporter (Zastawny, 1993) was significantly *increased* when co-transfected with an expression vector encoding a mutant p53 (p53¹⁷⁵[Arg→His]) cDNA. This response was localized to sequences between -106 to -40 in the MDR1 promoter, or possibly downstream of the transcription start site (between +128 to +700), since deletions including these regions dramatically decreased the effects of p53 cotransfections. Other studies found that p53 activation required only the minimal core promoter sequences of the MDR1 (Nguyen, 1994) and pgp1 (Zastawny, 1993) promoters. This activation was also seen using other p53 mutants (p53¹⁴³[Val→Ala], p53²⁴⁸[Arg→Trp], p53²⁷³[Arg→His], p53²⁸¹[Asp→Gly]) and other cell lines (Hep G2 and PLC/PRF/5 (human hepatocellular carcinoma), SK Hep (endothelial carcinoma), JAR (human choriocarcinoma) and MCF-7 (human breast cancer), and AuxB1 cells (Chinese hamster)). Interestingly, in mouse embryo fibroblasts (p53^{-/-}) derived from p53-deficient (p53^{-/-}) mice, mutant p53 proteins were able to induce MDR1-CAT activity up to 2–6 fold, indicating that the mutant proteins are not simply acting as dominant negative chelators of wild-type p53. In contrast, co-transfection of mutant p53 constructs into human p53⁻ H358 lung carcinoma cells, SW620 colon carcinoma cells, 2780 ovarian carcinoma cells or SW13 cells failed to stimulate expression of an MDR1-CAT construct (Goldsmith, 1995).

The role of wild-type p53 in Pgp regulation is equally paradoxical. Transfected wild type p53 had no effect on the MDR1-CAT reporter construct in NIH-3T3 (Chin, 1992) cells, dramatically *decreased* the expression of the MDR1-CAT reporter in SW13 cells, and stimulated activity of the -89/+53 MDR1-CAT construct in H358, SW620 and 2780 cells (Goldsmith, 1995). It should be noted, however, that all of the aforementioned studies evaluated the effect of p53 proteins on transfected Pgp promoter constructs. Recently, the effect of p53 on endogenous MDR1 activity has been evaluated. In one study, a dominant negative p53 expression vector was stably transfected into rodent H35 hepatoma cells that express Pgp and wild-type p53 (Thottassery, 1997). The levels of Pgp mRNA and protein were markedly elevated in these stable transfectants, and this increase was accom-

panied by an increase in resistance to MDR drugs. However, in a second study, wild-type p53 was stably introduced into the MDR cell line KB V200, which contains mutant p53 (Li, 1997). In this case, MDR1 expression was significantly increased, but toxicity in the presence of MDR drugs was also increased; it was suggested that the enhanced drug sensitivity was due to an increase in p53-mediated apoptosis.

Regardless of the effect of p53 on Pgp promoters, the mechanism of action appears to be indirect, since no p53 consensus binding sites have been identified within the p53-responsive region of MDR1 promoter. Moreover, purified p53 protein failed to bind the MDR1 promoter region required for activity although p53 binding sites were identified downstream of the MDR+1 site (Strauss, 1995). Therefore, the responses are likely to be mediated by interaction with other transcription factors or GTFs that are involved in Pgp transcription. It is probable that the various effects of p53 in different cell lines can be explained, at least in part, by differences in the baseline expression of Pgp in the cell, the complement of endogenous p53 (wild-type or mutant) and the presence or absence of these other factors or co-effectors of p53 activity.

Ras/Raf Activated Ras is a component of the signal transduction cascade involving Raf and MAP kinases (reviewed in Pelech, 1995). Several studies suggest that the MDR1 gene may be among the targets of this pathway. Transformation of rat liver epithelial cells with v-H-ras or v-raf resulted in both an increased expression of Pgp and increased resistance to MDR drugs and 2-acetylaminofluorene (Burt, 1988). In serum-starved NIH 3T3 cells, co-transfection of v-raf stimulated the expression of an MDR1-luciferase reporter construct ~10 fold (Cornwell, 1993b). It was also shown that co-transfection of a dominant-negative raf mutant blocked the serum stimulation of the reporter construct, both in NIH3T3 and Hep G2 cells. The transcriptional response to v-raf was mapped to the region between -69 and -41 in the MDR1 promoter, which includes the previously identified SP1 site; interestingly, a binding site for EGR-1 (which mediates activation by TPA (McCoy, 1995)) was found to overlap the SP1 site. While mutation of the EGR-1 site had no effect on stimulation by v-raf, a mutation that inhibited Sp1 binding at this site abolished v-raf-mediated activation, suggesting the involvement of SP1 in ras-activated induction of MDR1 transcription (Miltnerberger, 1995). Raf-1 kinase activated transcription of the rat *mdr1b* promoter

through an NF κ B binding site, while the introduction of a dominant-negative Raf-1 kinase inhibited promoter activation by insulin, indicating that the insulin effect is mediated by NF κ B through a Raf-1 signaling pathway (Zhou, 1997).

Inducible regulators

Stress induction of P-glycoprotein genes From the earliest studies of P-glycoprotein function and regulation, it has been suggested that Pgp is a 'stress-response gene' since its activity can be modulated by environmental adversity (reviewed in Fairchild, 1991). These inducers include heat shock, the surgical insult of partial hepatectomy, exposure to carcinogens including chemotherapeutics, and UV- and X-irradiation. Although the transcriptional events that underlie these responses are not completely understood, some progress has been made in this area.

Heat shock MDR1 mRNA levels were initially shown to be increased by heat shock in the renal adenocarcinoma cell-line HTB-46 (Chin, 1990). In this study, treatment with sodium arsenite and cadmium chloride elicited similar responses as exposure to heat, although it was not determined whether the induction was due to an increase in transcription. Subsequent investigation showed that heat shock could indeed increase transcription from an MDR1 reporter construct (Kioka, 1992). Interestingly, a previously identified putative heat-shock element, (HSE, -178 to -152) was not required for activation. Instead, a region described by deletions from -136 to -76 was required to elicit the promoter response, although oligonucleotides including this region did not appear to interact with the inducible heat shock factor (HSF) (Miyazaki, 1992). However, recent work has suggested that HSF may play a role in the regulation of MDR1 transcription in multi-drug resistant P388/M leukemia cells. These cells displayed constitutive HSF binding to MDR1 HSEs, even without heat shock, when compared to the parental drug-sensitive cells (Kim, 1997). Whether this interaction is functionally significant remains to be determined.

Carcinogens Most studies on the effects of carcinogens on P-glycoprotein expression have concentrated on the rat genes. These genes have been shown to be induced by a number of xenobiotics including 2,3,7,8-tetrachlorodibenzo-p-dioxin (Teeter, 1991a), 2-acetylaminofluorene (Gant, 1991; Schrenk, 1994; Hill, 1996; Lecureur, 1996) and 3-methylcholanthrene

(Chieli, 1994; Fardel, 1996a). Nuclear run-on studies established that the increase in rat *mdr1b* expression following exposure to these compounds was due to an increase in transcription (Gant, 1991). Although these carcinogens are known to modulate transcription through the aryl-hydrocarbon (Ah) receptor, it is unlikely that Ah acts on the *mdr1b* gene.

Recent work suggests that there may be differences in the responses of human and rat hepatocytes to carcinogens (Lecureur, 1996). In cultured rat hepatocytes, AAF elicited the expected increase in *mdr1a* mRNA levels, but under similar conditions in human hepatocytes there was no change in MDR1 or MDR2 expression. This was not merely due to the unresponsiveness of the human cells to AAF, as cytochromes P450 CYP1A1 and CYP1A2 were induced in both cases by the drug treatment.

Chemotherapeutics Considerable evidence has accumulated to indicate that the transcription of Pgp genes can be transiently induced in response to chemotherapeutics (Gekeler, 1988; Kohno, 1989; Chin, 1990; Chaudhary, 1993; Asakuno, 1994; Hu, 1995; Ohga, 1996; Schuetz, 1996). This was first reported in CCRF-CEM/ActD cells which were shown to exhibit increased steady state levels of Pgp RNA following short-term exposure to actinomycin D (Gekeler, 1988). The increase in P-glycoprotein mRNA upon treatment with chemotherapeutics is thought to be a transcriptional event, at least in the case of actinomycin-D induction, where the required sequences within the MDR1 promoter have been mapped to the -136 to -76 region that includes the inverted CCAAT element (Ohga, 1996). Notably, interaction of this site with a transcription factor believed (although not directly shown) to be YB-1 is increased in the presence of actinomycin D (Asakuno, 1994; Ohga, 1996).

Although it was initially assumed that only those drugs associated with the MDR phenotype would induce the expression of Pgp genes, more recent studies indicate that in a variety of cell lines derived from tumor types, Pgp transcription could also be induced by non-MDR drugs, such as antifolates, cisplatin and hydroxyurea (Chaudhary, 1993). In this study, induction of MDR 1 was associated with morphological indications of cell damage, suggesting that increased Pgp transcription may be part of a general cellular response to damaging agents.

The finding that P-glycoprotein gene expression can be induced by transient exposure to chemothera-

peutics has potential clinical significance. Recent *in vivo* experiments in human patients have shown that transient exposure to doxorubicin leads to a rapid (20–50 min) induction of MDR1 expression in lung metastases (Abolhoda, 1997). This observation may explain why a correlation between outcome and P-glycoprotein expression has been difficult to establish in solid tumors, since transient overexpression of P-glycoprotein induced by the MDR drug itself would not be detected in tumors subsequent to treatment. Further studies are required to determine whether the same mechanism(s) responsible for *in vitro* induction of Pgp genes by chemotherapeutics are also operative in human tumors.

Inflammation The acute-phase response is a general term for the complex changes that take place in mammals in response to inflammatory stimuli such as bacterial infection or burn injury. In the laboratory, this response is usually simulated in rodents by administration of bacterial lipopolysaccharide (LPS). In response to LPS, macrophages secrete inflammatory cytokines such as IL-1, IL-6, and TNF, which in turn act on the liver to induce a change in that organ's gene expression program, resulting in the synthesis of a range of acute-phase proteins (reviewed in Akira, 1992)).

Under acute-phase conditions, P-glycoprotein genes are induced in the liver (Nakatsukasa, 1993). Interestingly, at least two transcription factors that are known to be induced during the acute phase response have been shown to regulate P-glycoprotein gene expression in some cell lines. Studies carried out in a number of laboratories have shown that the C/EBP family member NF-IL6 can activate the mouse and human class I P-glycoprotein genes in transfection assays (Combates, 1994; Yu, 1995). We have shown that the homologous region in the hamster pgp1 promoter contains an NF-IL6 binding site that is also involved in activation and that activation through this site can be modulated by the binding of the glucocorticoid receptor (see below). These results suggest that this element may be important as a site of cross-talk between inflammatory signals mediated by cytokines through NF-IL6, and anti-inflammatory signals mediated by glucocorticoids through their receptor. Further studies are required to test this hypothesis.

It has recently been shown that NF κ B, another factor involved in the inflammatory response, can also regulate Pgp expression (Zhou, 1997). When cultured hepatoma cells were exposed to insulin, the rat *mdr1b* promoter was activated through an element including

sequences from –243 to –163. Further analysis revealed that an NF κ B binding site (–167 to –158) was required for this activation, and that insulin stimulated the binding of this factor to its cognate site. Moreover, NF κ B-mediated induction of the *mdr1b* promoter occurred via the Raf-1 kinase signaling pathway (see below).

UV-irradiation UV-irradiation was found to activate a human MDR1-CAT reporter construct approximately 20-fold following transfection into human KB cells (Uchiumi, 1993). Two elements, one extending from –136 to –76 and the second extending from +1 to +121, were found to be required for this activation. Recently, it has been shown that induction of MDR1 transcription is mediated by the inverted CCAAT box, and that downregulation of the CCAAT box binding protein, YB-1, decreases promoter response to UV. These results, together with the observation that MDR1 activation by chemotherapeutics may also be mediated by YB-1, suggest that YB-1 is directly involved in MDR1 activation by genotoxic stress (Ohga, 1998).

Hormones The progesterone receptor (PR) was the first steroid receptor to be shown to regulate P-glycoprotein transcription. The first indication that progesterone could influence Pgp expression came from the observation that the expression of the mouse class II gene, *mdr1b*, was increased in the secretory epithelium of the gravid uterus as compared to the non-pregnant uterus (Arceci, 1990). Transient transfection studies indicated that the progesterone response required DNA sequences within the first 100 bp of the first untranslated exon, but DNA-binding by PR within this region was not observed (Piekarz, 1993). Recent studies by the same laboratory would suggest that the activation of *mdr1b* by PR is actually an indirect effect. In this study, the increase in transcription was found to be mediated by sequences between –122 and –65. Mutations in binding sites for NF-IL6 and NF-Y found within this region decreased induction by progesterone, suggesting that these factors may be required for the observed effects (Mallick, 1997).

A number of studies have suggested a role for glucocorticoids in the regulation of P-glycoprotein gene transcription, but the responses are cell-type and gene-class dependent. Using the mouse hepatoma cell-lines Hepa 1-6 and hepalc1c, it was found that the synthetic glucocorticoid, dexamethasone, elicited an increase in the expression of *mdr1a* and *mdr1b*, but

not *mdr2* (Zhao, 1993). Nuclear run-on analysis indicated that this increase occurred at least in part at the transcriptional level and could be abrogated by the protein synthesis inhibitor cycloheximide, suggesting that GR was influencing this promoter through an indirect mechanism. A similar increase in human MDR1 RNA levels was observed in the HepG2 human hepatoma cell line. No increase was seen in the non-hepatoma mouse LMtk- and NIH3T3 cellline or the human HeLa cell line upon dexamethasone treatment, suggesting that the effect is hepatoma cellline specific.

In a similar study in rat H35 cells, sequences between -177 and -66 of the *mdr1b* promoter were found to be required for activation by dexamethasone. Since a glucocorticoid receptor (GR) binding site could not be identified within this region, it was proposed that, like the PR effect, the role of GR in this activation was indirect (Schuetz, 1995). In rat primary hepatocytes, however, dexamethasone treatment led to a decrease in *mdr1b* expression, although a post-transcriptional mechanism was proposed.

A glucocorticoid response element (GRE) has been identified by our laboratory in the hamster class I *p-gp1* gene (Egan, 1995). This site, between -96 and -83, mediates the repression of *p-gp1* transcription by GR in transient transfection assays in both DC-3F Chinese hamster lung cells and a human osteosarcoma cell-line (U2-OS). The GRE overlaps a binding site for NF-IL6, a member of the C/EBP family of transcription factors that has previously been shown to activate class I P-glycoprotein transcription (see above). It appears that GR represses *p-gp1* transcription by interfering with the actions of NF-IL6, as both sites are required for repression to occur. Interestingly, these elements are conserved in the human MDR1 promoter. MDR1 transcription can also be repressed by GR, suggesting that a similar mechanism may be involved.

The progesterone and glucocorticoid receptors are the first transcriptional regulators of P-glycoprotein transcription to be described that are amenable to modulation using readily available drugs. This, along with the fact that glucocorticoids are currently used in chemotherapy for tumors such as chronic lymphocytic leukemias, lymphomas, multiple myelomas and breast cancers, makes it critical to understand the role of these hormones in P-glycoprotein transcriptional regulation.

Differentiation agents Exposure to retinoic acid results in the differentiation of several neuronal (N)-type neuroblastoma cell lines (reviewed in Redfern, 1995).

This differentiation is accompanied by an increase in endogenous MDR1 mRNA levels (Bates, 1989) and the transcriptional activation of an MDR1 promoter/reporter construct, although the factors involved in transcriptional activation have not yet been identified (Ferrandis, 1994). Similarly, differentiation of colon cell lines with sodium butyrate is accompanied by an increase (20–40 fold) of MDR1 mRNA (Mickley, 1989) and a concomitant activation of MDR1 reporter constructs (Morrow, 1994; Jin, 1998). Activation by butyrate has been mapped to the inverted CCAAT element within the promoter and will be discussed in more detail later in this chapter.

The effect of differentiation agents on Pgp transcription is highly cell-specific. While many cells respond to sodium butyrate and retinoic acid by differentiation accompanied by an increase in P-glycoprotein expression, other cells either do not respond at all (SW-403, SW-1116, HT-29, WIDR), or increase P-glycoprotein expression in the absence of differentiation (K652 cells).

Regulation of Pgp transcription in MDR cells Although a number of DNA elements and transcription factors involved in the regulation of Pgp transcription have been identified, and many of these may be involved in gene expression in intrinsically resistant cells, the processes underlying the activation of Pgp genes in cells selected for acquired drug resistance has remained elusive. However, two studies have shed light on possible mechanisms of Pgp activation during the acquisition and maintenance of the MDR phenotype.

MED-1 Despite up to a 200-fold increase in steady state *p-gp1* mRNA levels between drug-sensitive and MDR Chinese hamster cells, transiently transfected *p-gp1* promoter/reporter constructs support equal levels of transcription in both cell types. However, stable integration of these same constructs into sensitive and MDR cell lines partially reproduce the overexpression phenotype (Ince, 1996). Under these conditions, mutation of a downstream *p-gp1* promoter element, MED-1, decreased transcription in MDR cells without affecting activity in drug-sensitive cells. This is the first report of a DNA element differentially regulated in drug-resistant cells. Moreover, it was the first indication that chromosomal integration, and by inference, chromatin structure, was involved in the regulation of Pgp genes. The role of chromatin in Pgp transcrip-

tion will be discussed in greater detail in the following section.

Gene rearrangement A t(4q;7q) translocation in an adriamycin-selected human cell line resulted in a hybrid mRNA containing sequences from both MDR-1 and a novel gene normally located on chromosome 4 (Mickley, 1997). This gene rearrangement resulted in the activation of MDR1 expression mediated by promoter sequences found within the translocated chromosome 4 DNA. Additional gene rearrangements have been identified in other MDR cell lines and in some patient tumors, suggesting that this may be a frequent mechanism for overexpression of otherwise silent Pgp genes in acquired drug resistance.

The role of chromatin in Pgp transcription

Until recently, the majority of transcription studies have focused only on the direct interaction of transcription factors with their cognate binding sites. Although this interplay between DNA elements and transcription factors occurs within the presence of a complex chromosomal architecture, there have been relatively few studies addressing the contribution of chromatin to transcriptional regulation. However, a heightened interest in this area has been spurred by the recent cloning of the histone modifying enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes with opposing effects on chromatin organization (reviewed in Struhl, 1998). HATs specifically catalyze the acetylation of the ϵ -amino group of lysine residues at the N-terminal domain of histones, weakening histone-DNA actions and leading to a destabilization of nucleosome structure ('open' chromatin), while HDACs remove acetylated residues leading to a more 'closed' chromatin configuration. It has been proposed that this restructuring of chromatin regulates accessibility of transcription factors to their DNA targets, whereby open chromatin allows for factor binding and closed chromatin does not.

Earlier studies indicated that the differentiation agent, sodium butyrate (NaB), activates MDR1 transcription through the inverted CCAAT element (Morrow, 1994). Although at the concentrations used NaB has a myriad of pleiotropic effects on cellular proteins, one of its known targets is HDAC. To determine whether inhibition of HDAC activity, and by inference alteration of chromatin structure, is the mechanism by which butyrate activates the MDR1 promoter, our lab-

oratory has directly evaluated the effect of the highly specific HDAC inhibitor, trichostatin (TSA) on MDR1 activity (Jin, 1998). Both the endogenous MDR1 promoter as well as MDR1 promoter/reporter constructs were activated to a similar extent and within a similar time frame by both TSA and NaB. Moreover, activation was also achieved through the overexpression of pCAF, a transcription factor with intrinsic HAT activity. Lastly, we have shown that activation by HDAC inhibitors or P/CAF is dependent on the transcription factor NF-Y, which recruits P/CAF to the inverted CCAAT box in the MDR1 promoter. It is intriguing to speculate that other CCAAT box-mediated responses (i.e. induction by UV-irradiation and chemotherapeutics) may also work through a HAT/HDAC-dependent mechanism.

While the concentration used to activate the MDR1 promoter in SW620 colon carcinoma seems high (2 mM), the endogenous concentration of butyrate in the normal human colon can be as high as 20 mM and is believed play a role in regulating colon epithelial cell maturation. Mature colon epithelial cells have high levels of expression of MDR1 relative to most other cell types, and it is interesting to speculate that this high intrinsic expression could be modulated by the effect of butyrate on HAT/HDAC activities. Moreover, these results compel an assessment of whether phenylbutyrate, which is presently included in several chemotherapeutic protocols, activates endogenous MDR1 transcription in these tumors, possibly leading to enhanced drug resistance.

Conclusions

Over the past two decades, intensive research has been directed at understanding the function and regulation of Pgp genes in normal tissues and drug-resistant tumor cells. What has emerged from these studies is an appreciation for the complexity of factors, both intrinsic and environmental, that interact to regulate Pgp levels in a given cell type under a given set of conditions. With this knowledge in hand, future studies will be directed at finding ways in which to modulate Pgp expression in order to prevent or overcome the development of Pgp-mediated drug resistance in human tumors.

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