Gene Expression and Its Physiological Control in Disease and Development

Studies on the Human PDGF-B Gene and Tumour Hypoxia

BY

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ABSTRACT

Strict control of gene expression is essential during development and in response to physiological stimuli. This thesis describes the functional characterisation of the gene regulatory mechanisms controlling the expression of the potent human growth factor Platelet Derived Growth Factor B gene, in a cell type specific context and in response to low oxygen tension. In addition, analysis of hypoxia in neuroblastoma indicates a role during tumour differentiation.

Initially, a promoter-specific enhancer system controlling the expression of PDGF-B was characterised in placentally derived choriocarcinoma cells. The specificity of this enhancer promoter interaction was shown to be dependent on specific sequence elements identified in both the promoter and enhancer regions. It was then shown that the activity of the PDGF-B promoter is controlled via modulation of histone acetylation status in a cell type specific manner and furthermore, that one role of its enhancer could be to regulate transcription via alterations in acetylation status at the promoter.

PDGF-B expression was then shown to be controlled by hypoxia in a biphasic manner in bladder carcinoma cells. An initial induction was followed by repression of transcription following chronic hypoxia. The biphasic response was shown to be dependent on glucose levels and uniquely amongst hypoxia regulated genes studied so far, PDGF-B expression was shown to be repressed by low glucose.

Finally, detailed *in vivo* and *in vitro* analysis revealed that the major form of differentiation in childhood neuroblastoma is towards chromaffin-like rather than ganglionic lineages. This type of differentiation did not correlate with disease progression but was suggested to be dependent on tumour hypoxia, since chromaffin differentiation markers co-localised with markers of tumour hypoxia in both clinical samples and xenogenic tumours.

In conclusion, the work presented in this thesis has identified several novel, highly specific gene regulatory mechanisms that are involved in development, the response to physiological stimuli and in disease progression.

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ABBREVIATIONS

ARNT         Aryl hydrocarbon receptor nuclear translocator
CAT          Chloramphenicol acetyl transferase
CTD          Carboxy terminal domain
Dnmt         DNA methyl transferase
EDC          Enhancer dependent cis-coactivator
EDCse        EDC specificity element
EMSA         Electro-mobility shift assay
EPAS         Endothelial per-arnt-sim
GAP          Growth associated protein
GTF          General transcription factor
HAT          Histone acetyl transferase
HDAC         Histone deacetylase
HIF          Hypoxia inducible factor
HRE          HIF response element
Inr          Initiator
IGF          Insulin-like growth factor
kb           kilobase
MeCP2        Methyl-CpG-binding protein 2
ORF          Open reading frame
PDGF         Platelet derived growth factor
PIC          Pre-initiation complex
RNA Pol       RNA polymerase
SIF          Small intensely fluorescent
SNS          Sympathetic nervous system
TAF          TBP associated factor
TBP          TATA-binding protein
TRF          TBP related factor
TSA          Trichostatin A
UPE          Upstream promoter element
UTR          Untranslated region
VEGF         Vascular endothelial growth factor
INTRODUCTION

One of the most fascinating facts in biology is that one single cell, the fertilised egg, will give rise to all the different cell-types constituting the adult animal. The fertilised egg will differentiate into a large number of very specialised cell-types, such as neurons, red blood cells and muscle cells. The morphological and functional differences between these cell-types are largely dependent on the subset of genes that are expressed, or were expressed during the development of a certain cell lineage. The fertilised egg and the vast majority of its descendant cells contain the same set of genes. Generally, there is no loss, or addition of genes during development. Instead, it is the extremely intricate control of gene expression that directs development. Certain genes have to be activated, expressed at a specific level and then perhaps repressed again. Different genes have to be expressed at different time-points and in different cell-types for development to proceed and in terminally differentiated cells, gene expression is also strictly regulated. A multitude of different factors influence gene expression. Signalling within and between cells, as well as environmental factors such as pH, temperature and oxygen levels all influence gene expression.

The control of gene expression can take place at different levels. The function of the protein product can be modified, by phosphorylation or glycosylation for example and the stability of the protein may also be regulated. Earlier on, processing of the mRNA is also an important regulatory step. The major level at which gene expression is controlled, however, is at the transcriptional level; the initial step in gene expression. Transcription is regulated in many ways during development and in the adult. The transcription of certain genes can be activated or actively silenced and the expression level modulated by different stimuli. A strict control is crucial, since aberrant responses to stimuli will alter gene expression, which could lead to diseases such as cancer.

In this thesis, I describe the characterisation of some of the mechanisms that control the cell-type specific transcriptional regulation of the human Platelet Derived Growth Factor B (PDGF-B) gene. The transcription of the potent growth factor PDGF-B has to be strictly regulated both temporally and spatially during development. This thesis shows that very specific mechanisms are utilised to control PDGF-B transcription in placentally derived cells, involving both specific DNA elements and regulation at the chromatin level. The transcriptional response of PDGF-B
to hypoxia, low oxygen tension, is also analysed and hypoxia is also shown to be involved in the differentiation of neuroblastoma tumours.

THE PROCESS OF TRANSCRIPTION

The genome encodes all the genes required for the development and function of the organism. It is essential that specific genes are expressed in the right cell type during the different phases of development and in addition, that the cells can respond to external and internal stimuli by altering the expression of specific genes. It is crucial, therefore, that the expression of the genome is strictly controlled, both during development and as a function of diverse stimuli in the adult organism. According to recent estimates the human genome carries of the order of 25 000 to 50 000 genes, implying that only a very small proportion of the genome consists of gene coding sequence [1]. This means that there is a large opportunity for non-coding sequences to be used for controlling the expression of genes and other chromosomal processes. It should be pointed out that keeping genes silent could be regarded as being as important as the activation of certain genes. In the processes regulating gene expression, the packing of DNA into chromatin plays a significant role, which will be discussed in more detail below. Furthermore, it has been estimated that a minimum of 1000 of the perhaps 50 000 human genes are involved in controlling gene expression, underscoring the importance of this process [2]. Although gene expression is also regulated at the post-transcriptional level by processes such as splicing, mRNA-stability, translation and protein modification, it is clear that most of the regulation of gene expression occurs at the level of transcription.

Transcription is the process during which the information encoded by the DNA is converted to an RNA intermediate that is then processed and translated into protein, or alternatively, the RNA itself can be functional in some cases. The basal features of transcription are very well conserved within eukaryotes, but there is a great diversity of ways in which transcription is regulated. In eukaryotes there are three different related RNA polymerases that carry out transcription. RNA polymerase I transcribes ribosomal RNAs (5.8S, 18S and 28S). It actually transcribes the bulk of the total RNA in the cell, but the requirements for strict regulation are different compared to RNA polymerase II transcribed genes. RNA polymerase II transcribes all mRNA encoding genes, as well as some small nuclear RNAs. Finally, RNA polymerase III transcribes tRNAs, 5S RNA, some small nuclear RNAs as well as small cytoplasmic RNAs. All three polymerase complexes contain the same basic catalytic sub-unit and carry
out the same job, that is the initiation and elongation of transcription. They differ, however, in the requirement for necessary factors and cis-elements. The following section will concentrate on transcription by RNA polymerase II (Pol II). The transcription cycle can be broadly divided into three parts: initiation, elongation and termination. Initiation is the process whereby the polymerase and associated factors recognise and bind to a promoter, which is often the step at which regulation occurs.

**Basal transcription**
The control of transcription can be divided into two levels. Basal transcription is defined as a low level of accurately and directionally initiated transcription, whereas in regulated transcription, the rate of basal transcription is modulated. Basal transcription is dependent on interaction between a few well-defined cis-elements and a number of general transcription factors that will be briefly described below.

**Pol II gene core promoters**
In order for transcription to be initiated, the polymerase complex has to recognise a specific promoter sequence located 5′ of the transcribed region. The promoter often consists of an AT-rich TATA box sequence located approximately 25 base pairs upstream of the transcription start site. The TATA box consists of around 8 T:A base pairs flanked by G/C base pairs. A symmetric TATA-box alone will not, however, specify the direction of transcription [3]. TATA-less promoters have also been characterised, for which transcription initiation is dependent on an initiator (Inr) sequence with the consensus YYCAYYYYY, (Y=T/C) which is usually located at the transcriptional initiation site (denoted +1) [4]. An Inr-containing promoter will initiate accurate transcription at the right position without a TATA-box [5]. There are also examples of promoters containing both a TATA-box and one or more Inr-sequences. In these cases, the Inrs and the TATA-box cooperate, which has been shown to increase transcriptional activity [6]. Some promoters contain neither a TATA-box, nor an Inr consensus. These promoters depend on other sequences, such as GC-rich boxes or proximal sequence element (PSE) [7]. The Pol II promoter also contains a Cap-site located at the transcription start site (+1)

**Components of the Pre-initiation Complex**
The core promoter described above is sufficient for recognition by an RNA polymerase complex, which will then form a so-called pre-initiation complex (PIC) on the promoter that will subsequently initiate transcription. The PIC, in addition to the polymerase, consists of a number of general
transcription factors (GTFs) that have been characterised both genetically and biochemically. TFIID is the only general transcription factor with specific DNA binding capacity. It consists of TATA binding protein (TBP) and a number of TBP associated factors (TAFs). TBP is a highly conserved saddle-shaped protein with specific affinity for the TATA-box sequence [8]. Most studies indicate that TBP is also essential for the transcription from TATA-less promoters. Recently, tissue specific TBP related factors 1 and 2 (TRF) have also been described [9-11].

The other TFIID components, the TAFs, are either ubiquitous, specific for the TFIID complex of a subset of genes [12] or cell type-specific [13]. The TAFs are thought to interact with activators and/or repressors bound to upstream elements, as well as different co-regulators, thereby mediating regulation of the PIC function, although they seem to be dispensable for basal transcription in vitro. TAFs are also thought to interact directly with other components of the basal machinery to direct promoter specificity [14, 15]. In this way, the TAF composition of different TFIID complexes provides an important target for regulatory factors and processes [2].

The next component of the PIC, TFIIA, has been shown to function in stabilising the TBP/TATA complex, possibly via interaction with the TAFs. TFIIIB specifies the start site selection by the polymerase, 30 bp downstream of the TATA box and TFIIF, together with TFIIIB, recruits the polymerase into the PIC. The RNA polymerase II is a large multi-subunit complex. The largest sub-unit carries a conserved carboxy-terminal domain (CTD) with a number of amino acid repeats that have the potential to be phosphorylated. These have been shown to act as target for a subset of activators. TFIIE and TFIIH, are involved in the switch from initiation to elongation. TFIIE is necessary for TFIIH recruitment and TFIIH harbours a kinase activity that phosphorylates the Pol II CTD, thereby reducing the affinity of Pol II for TFIID, which enables elongation to proceed. TFIIH also has an ATP-dependent helicase activity that will unwind the DNA at the promoter, facilitating elongation. In total, around 40 different polypeptides make up the PIC. For a more detailed description of the function of the GTFs, see [16].

The GTFs were earlier assumed to sequentially assemble within the PIC forming at the promoter, but this traditional view has been questioned. It has been shown that the PIC is generally present as a holo-enzyme complex that will interact directly with the promoter [17] (fig. 1). A number of different holo-enzyme complexes have been characterised and shown to include different subsets of the GTFs and also other multi-protein complexes such as mediators, transcriptional co-activators and chromatin remodelling complexes [18]. Despite the heterogeneity of these holo-
complexes, it is clear that they do not contain TFIID, indicating that the assembly of the PIC requires at least a two step process with initial TFIID binding to the TATA-box, followed by recruitment of the rest of the PIC. The appealing feature of a holo-enzyme scenario compared to the stepwise assembly model is that it would seem to be favoured because of the limiting amount of constituent factors in the nucleus and the need for a quick response to activators. This, however, also brings up questions about recycling of the PICs and constituents of the elongation complex.

Figure 1. Sequential assembly versus holo-enzyme view of the PIC
A viable compromise between the opposing models could be a holo-complex consisting of a limited number of exchangeable modules. This would give the advantage of the holo-complex but at the same time give the opportunity of greater regulatory flexibility.

There are also different models of how the transcription of a gene in the nuclear setting is envisaged. Either the PIC is localised to the promoter by random diffusion and DNA/protein and protein/protein interaction, or the holo-enzyme could be regarded as a huge transcription factory, which may be present at distinct locations in the nucleus. This “factory” could then recruit the genes to be transcribed, possibly by specific interactions involving regulatory cis-elements and regulatory factors. Indeed, some reports indicate that active transcription is confined to a few thousand more or less distinct foci in the nucleus [19, 20]. It is becoming increasingly clear that the topography of the nucleus involving chromatin domains, nuclear scaffold and distinct processing compartments analogous to the nucleolus are fundamental for diverse nuclear processes. It remains to be determined, however, to what extent nuclear topography is involved in the regulation of transcription.

After the PIC has been released from the core promoter elongation proceeds until it is terminated, around which time the transcript is polyadenylated and further processed. A multitude of factors are necessary for elongation, termination and RNA processing, but transcription is mainly regulated at the initiation/promoter clearance level. I will therefore discuss the cis-elements and protein factors that are involved in regulated transcription.

**Regulated transcription**

As mentioned above, the core promoter and PIC complex are only capable of promoting a low basal level of transcription, which is initiated at the correct position. Further sequence elements and protein factors are necessary to regulate the level of transcription and to equip the gene with the potential to be regulated temporally and spatially during development and by external stimuli.

There are a number of more or less distinct types of regulatory cis elements: Upstream promoter elements (UPEs) and enhancers/silencers and Insulators. UPEs are typically located within 200 base pairs upstream of the transcription start site. They have the capacity to bind regulatory factors that either promote or inhibit transcription, for example the Sp1 transcription factor. These factors, in some cases, have the potential to bind more distally located enhancer or repressor elements [21]. Enhancers/silencers can be described as DNA elements that bind regulatory protein factors that then
interact with the transcriptional machinery at the core promoter. Enhancers were initially defined as elements that cannot promote transcription initiation themselves but work via increasing the transcription from a promoter, in a distance and orientation independent manner. Silencers, conversely, are defined as elements that decrease transcription from a promoter. Insulators will control the interaction between other regulatory elements as discussed below.

Enhancer cis-elements
Numerous models have tried to explain the functional mechanism of enhancers. What is definitely clear is that one function of enhancers is to bind regulatory protein factors. Enhancers are often modular, containing a number of short recognition sequences that will attract DNA-binding protein factors [22]. Some cis-elements are recognised by many different factors that compete or synergise in their binding, thereby yielding complex expression patterns. The availability of active transcription factors will in part determine tissue specific expression of a certain gene. In this way, developmental regulation of transcription factor expression will regulate the expression of downstream genes [23]. These factors could either activate or repress transcription, thereby yielding a combined specific output on the transcription from the promoter.

Enhancer associated protein factors.
A large number of families of transcription factors that interact with enhancer elements have been described. They usually consist of a DNA binding motif and an activation domain that will somehow interact with the transcription machinery and frequently also include a dimerisation domain. The DNA binding domains have been divided into classes such as helix-turn-helix or zinc-finger containing, whereas the activation domains are classified based on constituent amino acids rather than functional properties. Other, additional factors can interact with these DNA binding factors and act as adapters, co-activators or co-repressors of the activation signal. One class of these intermediate factors is the mediator complex in yeast, which functions in vivo by interacting with the Pol II CTD [24]. In mammals, TAFs as well as positive co-factors, homologous to the mediator complex, appear to be crucial for activation by some enhancer-bound transcription factors. A large array of different co-activators and co-repressors have been characterised and shown to be associated with DNA-binding activators and the basal transcription machinery, as well as with other multi-protein complexes involved in regulating chromatin structure [25-27]. Indeed, many co-activators have been shown to exert their function
by making the chromatin structure at the promoter more accessible, as will be further discussed below.

**Enhancer mechanisms**

In most cases, the actual mode of activation by DNA-binding activators is still unknown, but it is generally believed that some activation domains interact with the general transcription machinery, either directly, or via adapter proteins, thereby stabilising the PIC on the core promoter. This stabilisation of the PIC will then increase the rate of transcription from the promoter [28]. Most studies point to TBP/TFIID binding as the rate-limiting step of transcription [29]. A few recent reports, however, suggest that the role of enhancers would rather be to increase the probability of transcription of a gene within a given cell [30, 31]. The observed increase in rate would then reflect that the analysis is performed on a population of cells. This on/off switch mode of enhancer action, however, leaves little space for regulatory flexibility. An additional problem would appear if the gene product would be required to function intra-cellularly. An on/off mode of regulation would then make little sense from the perspective of the individual cell. Another function of enhancers could be to direct a gene to a location in the nucleus where transcription is likely to occur, as discussed above. This mode of enhancer action becomes more plausible assuming that the polymerase acts as a localised holo-enzyme that recruits genes for transcription. The enhancer-bound activators may also function by changing the chromatin structure at the promoter, as will be further discussed below.

The question then emerges of how the activation signal is transmitted from the distal enhancer to the actual site of transcription. Several models have been proposed and the most widely accepted assumes that the DNA stretch between the enhancer and promoter will loop out, thereby enabling direct protein/protein interaction between the enhancer-bound transcription factor and the PIC. This model is supported by the observation of DNA loops in electron micrographs [32, 33] and is also consistent with the demonstration that certain enhancers can activate transcription in trans when promoter and enhancer are located on separate, non-covalently linked plasmids [34].

An alternative, or complementing, model of enhancer tracking along the DNA has also been described [35]. This model has gained support from the recent discovery of a specific class of factors that are loaded onto the DNA in a directional manner. It could then slide along the DNA until it reached the PIC at the promoter. One obvious complication with this model is that such a sliding-clamp transcription factor can be assumed to be able to only interact with the most proximal promoter. It is also difficult to imagine
how this sliding would go on uninterrupted for several kilobases, without being disturbed by other DNA-binding factors or tight chromatin structure.

A third model; the facilitated tracking model, combines the two previous ones to some extent [36]. Facilitated tracking suggests that the enhancer-bound protein factor makes small loops towards the target promoter, transiently contacting the intervening DNA. This scanning is terminated when the factor has reached the promoter and a stable loop is formed. Another potential model for enhancer activation outlines a scenario where the enhancer initially binds a sequence-specific factor, which will then attract other non-sequence specific facilitator proteins, thereby nucleating a transmission of a specific nucleoprotein structure extending from the enhancer [37]. This could act to control the transcriptional potential of an entire locus. This scenario is interesting, since it could explain the function of boundary elements, which is not so easily accommodated by the looping model of enhancer function.

Limitations of classical enhancers
Enhancers can be located at a very large distance from their target promoters, upstream, downstream or even within the transcription unit. The classical definition of an enhancer also specifies that it can work irrespective of orientation, that is, the sequence can be flipped without affecting the activating potential. The classical enhancer is also capable of activating any linked promoter [38] (fig. 2). The ability to activate heterologous promoters would imply that the enhancer exerts its effect on components of the transcription complex found at all promoters. A closer look at the definition of the classical enhancer reveals that such elements seem rather limited. An enhancer functioning in a genomic situation has to deal with additional problems not encountered in the simple experimental systems that have been used to characterise enhancer function. If all enhancers were capable of activating any promoter, a regulatory chaos could be expected. In many instances, genes are clustered which could lead to inappropriate action of enhancers on non-target promoters.

This problem has been resolved during evolution in a number of ways (fig. 2). In some cases, insulators demarcate chromosomal domains within which enhancers can act, while excluding interactions with promoters outside of the domain [39]. In other cases, the enhancer could have equal affinity for several promoters, but the promoters may be made selectively inaccessible to activation by the enhancer, in a developmental specific manner for example. An enhancer could also have different affinities for promoters, so that one promoter is preferentially activated, but another one could be activated to a lesser extent [40]. In the case of promoters
competing for the same enhancer, it has been shown that the promoter with the strongest TATA consensus will be preferentially activated [41]. It has indeed been shown that many enhancers activate a promoter in a cluster even though it is not the most enhancer-proximal one, but if this target promoter is experimentally deleted the enhancer can activate other promoters. Several cases have also been described where the enhancer/promoter interactions are specific, so that the enhancer can activate only its endogenous target promoter, for example the Drosophila goosberry locus [42], the dpp locus [43] and the rat carbamyl phosphate synthetase I gene [44]. These enhancer/promoter interactions are probably dependent on specific mechanisms that require additional factors apart from the general transcription machinery.

Figure 2. Classical versus promoter-specific enhancer action.
A) According to the classical definition an enhancer should be capable of activating any promoter in a distance and orientation independent manner.
B) This interaction is envisaged to involve the basal transcription machinery present at all promoters.
C) A promoter-specific enhancer activates only its cognate promoter (Px) and fails to activate other promoters.
P: promoter, E: enhancer
CHROMATIN AND GENE EXPRESSION

Stepping further into the complexity of the genome, it becomes apparent that the chromatin environment will also greatly affect gene expression. The genome is not simply a long stretch of DNA containing all the information for the inherent regulation of its expression. Rather, the DNA is packed into nucleosomes, which are further packed into higher order chromatin structure. Chromatin can be viewed as having a general repressive role that is necessary to control the large genome. Transcriptional activating mechanisms would then counteract this default repressed state. The organisation of the genome in the nucleus, with regions attached to the matrix and intervening loops, is also likely to affect gene expression. The simple modification of the primary sequence by methylation of cytosines is another mechanism that may strongly influence the ability of a gene to be expressed.

Many transcriptional co-activators have been shown to exert their function by making the chromatin more accessible and factors that increase the accessibility of chromatin have been shown to be integral parts of the basal transcription machinery. The ATP-dependent nucleosome remodelling complex SWI/SNF, which is tightly associated with the yeast RNA Pol II CTD is one such factor [45]. A number of SWI/SNF-related chromatin remodelling complexes have also been shown to be important co-regulators of transcription in mammals [46]. Furthermore, heterochromatin-like complexes that stably repress gene expression, partly by excluding activators, are important during development, as well as in the adult organism [47]. Taken together, the chromatin structure will modulate expression of the genome and a central theme in the control of transcription is to regulate the chromatin structure, either by making genes accessible for expression, or by keeping genes repressed to avoid leakage from basal promoter activity. Given that a majority of the genome is not supposed to be expressed and that some factors necessary for transcription may be limiting, it is necessary to have a system precluding inactive regions from factor binding [48].

A general model that describes the role of chromatin alterations in the activation of a gene, implies a multi step process. Initially a sequence-specific transcription factor is envisaged to bind chromatin DNA. This initial binding can be regulated by cell-type specific positioning of nucleosomes. The bound activator will then recruit co-activator complexes that modify histone acetylation status, as discussed below and/or remodel chromatin in an ATP-dependent manner. This will consequently result in facilitated binding of other activating factors and finally the PIC itself,
reviewed in [2]. In this context, it should be pointed out that chromatin remodelling and actual transcription seem to be separable events, so that a promoter can be in an activated chromatin state without being actually transcribed. For the purpose of this thesis I will concentrate on discussing the roles of DNA methylation and histone acetylation status in the regulation of transcription.

**DNA methylation and transcription**

The addition of methyl groups to the C-5 position on the cytosines of DNA is found in most higher eukaryotes. DNA methylation has been hypothesised to be an evolutionary adaptation to control parasitic sequences in the genome. A large portion of the mammalian genome consists of repetitive sequences and retroviral elements. Methylation of these sequences will prevent transcription, thereby limiting the spreading of these elements in the genome [49]. Another suggestion is that methylation functions in the control of gene expression during development of multi-cellular organisms. Programmed methylation and demethylation of regulatory sequences would then be important to direct developmental programmes [50].

Regardless of which explanation is most correct, there is no doubt that DNA methylation has a strong effect on gene expression. Methylation status has, for example, been implicated in the parental specific expression of imprinted genes [51]. Furthermore, many tumours and cultured cell-lines have been shown to carry growth control genes that are aberrantly repressed by excessive DNA methylation [52, 53]. Methylation is carried out by a class of enzymes displaying DNA methyltransferase activity. DNA methyl-transferase 1 (Dnmt1) is mainly a maintenance methylase, with preference for hemi-methylated DNA, while Dnmt 2 and 3 have *de novo* methyltransferase activity. There are also a few reports of factors with demethylating activity, reviewed in [54].

Methylation occurs almost exclusively on cytosines within CpG dinucleotides. Shortly after fertilisation, the bulk of these are demethylated and a different methylation pattern is established after implantation. A large proportion of all genes have regions of high CpG density in close association with their promoter regions. These so-called “CpG islands” are usually unmethylated, although this pattern can be disrupted during long term *in vitro* culture or during tumourigenesis [55]. The repressive effect of methylation can be attributed to different properties. Methylation can directly inhibit binding of sequence-specific DNA binding proteins that contain a CpG in their recognition sequence. One such example is the basic helix-loop-helix transcription factor HIF-1 that is described in some detail.
below [56]. Other transcription factors like Sp1 are indifferent to CpG methylation.

Methylated CpGs are specifically recognised by methyl-CpG-binding proteins like MeCP2 and MBD 1, 2 and 3, which contain the same conserved binding domain (MBD) [54]. Binding of MeCP2 to a methylated sequence will repress transcription by competing out binding of activating factors, or by recruitment of repressive proteins complexes containing histone deacetylases (HDACs) that will alter chromatin structure [57, 58], which will be further discussed below. There are also examples of non-HDAC dependent repression by MBD proteins [59]. It is not really clear how the different levels of repression synergise, or which event is dominant.

**Histone acetylation status and transcription**

DNA is generally packed in nucleosomes, which consist of a histone octamer with 146 bp of DNA wrapped around, constituting the nucleosome core particle, separated by linker DNA. Packaging of DNA into nucleosomes has a general repressive effect on transcription compared to naked DNA. In order for initiation to occur, the nucleosome structure at the core promoter has to be altered so that the PIC can bind. The general repressive role of nucleosomes has recently been called into question by the observation that partial depletion of histones in yeast did not correlate with a general increase in transcription [60]. Instead, the response appeared to be gene specific with some loci actually being repressed following histone depletion, suggesting a more dynamic role for nucleosomal chromatin in the regulation of gene expression.

The histone octamer is composed of two molecules each of histones H2A, H2B, H3 and H4. Histone H1 is not part of the core nucleosome but interacts with linker DNA and the core nucleosomes, possibly to enable higher order packing. The histones are evolutionary well-conserved, arginine and lysine rich proteins with non-sequence specific DNA binding affinity. All four histones contain a globular domain responsible for DNA/histone and histone/histone interactions and an N-terminal charged tail that contains the majority of the lysines. These tail regions are the sites of post-transcriptional modifications such as phosphorylation and acetylation. The addition of acetyl groups to the lysines neutralises the positive charge of the histone tails. Nucleosome structure is thoroughly described by Wolffe 1992 [61]. Hyper-acetylation of histones increases the regional sensitivity to DNaseI, indicating a looser nucleosome conformation [62]. Hyper-acetylation has been shown to be associated with active transcription, whereas hypo-acetylated histones are associated with transcriptional silencing, reviewed in [63], although there are exceptions to
this general rule.

The observed transcriptional activation is probably due to that increased acetylation will decrease the association between histones and DNA and between nucleosomes themselves, thereby increasing the accessibility for DNA-binding factors [64]. Other reports suggest a role for histone modifications in gene regulation that is independent of accessibility. The TFIID component TAF250 contains a double bromo-domain motif, which has been shown to specifically recognise and bind to the acetylated tail of histone H4, but not the non-acetylated form [65]. This indicates that TFIID may require an acetylation-modified nucleosome to bind, rather than nucleosome free DNA. It has also been suggested that specific combinations of histone tail modifications such as acetylation, methylation and phosphorylation could be the basis of a “code” that specifies factor binding [66]. It is also possible that the acetylation state of nucleosomes controls higher order chromatin packing, since acetylation state does not seem to affect the core nucleosome particle in the same way as the ATP-dependent remodelling complexes do.

**Histone acetyl transferases**

The acetylation status of histones is a function of the equilibrium between proteins with acetylase activity and deacetylase activity. Histone acetyl transferases, or HATs, were originally identified as transcriptional co-activators. Examples of proteins with HAT activity are GCN5 [67], TAFII250 [68], p300/CBP [69], ACTR [70], SRC1 [71] and pCAF [72]. It is intriguing that among these nuclear HATs, we find a TAF that is a component of the basal transcription machinery. The different HATs have specific affinities for different substrates, both in terms of histone sub-units and individual lysines. Furthermore, the different HATs are often associated with non-HAT proteins that modulate their activity, or are part of large complexes containing multiple HAT activities [73]. Many of the HAT proteins are sub-units of large activation complexes, like SAGA [74], some of which work in concert with chromatin remodelling complexes like SWI/SNF [75]. This fact ensures that HAT-containing co-activator complexes can elicit a complex and specific response in an *in vivo* context. A large number of sequence-specific activator proteins have been shown to recruit co-activators with HAT activity, for example NFκ-B [76], HNF4 [77] and HIF-1 [78]. The co-activator p300/CBP has also been found to be tightly associated with RNA Pol II holoenzyme, suggesting a more general role in transcriptional activation [79]. It is still unclear, however, whether the activating potential of the HATs is solely due to acetylation of histones. The HATs do not interact with histones only and several other proteins important for transcriptional regulation have
been shown to be targets for acetylation, including the tumour suppressor/transcriptional regulator p53 [80], the transcription factor GATA-1 [81] and the GTFs, TFIIE and TFIIF [82].

**Histone deacetylases**

Enzymes that deacetylate histones are referred to as histone deacetylases or HDACs. Three different classes of human histone deacetylases have been characterised: HDACs homologous to the yeast Rpd-3 enzyme, HDACs homologous to yeast Hda-1 proteins and NAD+ dependent HDACs homologous to yeast Sir2. Recent estimates indicate that the family of human HDACs is growing and could consist of as many as 18 different members [83]. As with the HATs, the different HDACs display distinct specificities for particular lysines. HDACs have been shown to interact with transcriptional repressors such as sin3 [84], MeCP2 [57, 58] and the initiator binding factor YY1 [85], thereby mediating repression of transcription. A number of specific inhibitors of the Rpd3 and Hda1 related HDACs have been characterised, including sodium butyrate, trapoxin and trichostatin A that can be used to modulate acetylation status in vivo and in vitro [86]. These inhibitors affect cell-cycle progression, apoptosis and proliferation by activating a subset of genes. It should be pointed out that HDAC inhibitors also repress transcription in some cases [87, 88].

HATs and HDACs are not intrinsically DNA binding, but rather they are recruited to sites in chromatin by protein/protein interactions. Studies in yeast cells lacking specific HDACs indicate that the expression of specific subsets of genes is altered [89]. This raises the question of whether changes in acetylation status have general or gene-specific effects. A general targeting to promoters is supported by the fact that HAT activities associate with components of the PIC/holo-enzyme. The sensitivity of individual promoters to alterations in acetylation status would then dictate the degree of activation/repression. It seems that HDAC/HAT activity can also be targeted to specific promoters by activators/repressors. It is unclear how far the effect on histone acetylation spreads from the target site and whether a single nucleosome or several nucleosomes spanning thousands of base pairs are modified.

It is clear that chromatin structure, protein factors and regulatory DNA sequences synergise to create a specific output of expression as a consequence of developmental programming or physiological signalling. Our model system for analysing the effects of chromatin structure, regulatory elements and physiological signals on the tight control of gene expression has been the human platelet derived growth factor gene.
PLATELET DERIVED GROWTH FACTOR

Growth factors are proteins that modulate cell proliferation and/or differentiation. In most cases, growth factors influence neighbouring cells in a paracrine fashion, or even the very cell that produced the factor, which is termed autocrine stimulation. Growth factors mediate their responses by binding to specific receptors, thereby eliciting an intracellular signalling cascade that results in alterations in cellular behaviour, altered gene expression patterns and ultimately, in increased proliferation or differentiation. It is of great importance that the expression and secretion of growth factors are strictly regulated during development and in the adult organism. Aberrant growth factor or receptor expression can cause developmental defects and proliferative disorders including cancer. In order to control growth factor gene expression, intricate regulatory mechanisms have evolved that will ensure correct regulation during development and by diverse physiological stimuli.

The potent human growth factor, platelet derived growth factor, (PDGF), was first isolated as the main serum mitogen for fibroblast and smooth muscle cell proliferation [90]. It was shown to be released from platelets in response to wounding. The isolated factor was found to be a dimeric peptide consisting of A and/or B chains. A third factor, PDGF-C, has recently been described [91], but it is unclear whether it can heterodimerise with the A or B chain proteins. The PDGFs along with vascular endothelial growth factors VEGFs and placental growth factor (PIGF) belong to the same family of cysteine knot growth factors, characterised by conserved cysteine residues that are involved in dimerisation via disulfide bonds. PDGF has since been found to have numerous roles during development and in normal physiological processes, as well as in pathological conditions [92]. The interest in PDGF was further boosted when the B-chain gene was shown to be the cellular equivalent of the transforming oncogene, v-sis, of simian sarcoma virus, suggesting a role in malignant transformation [93].

PDGF structure and function

The 30 kD PDGF dimer can function either as a B-chain homodimer, an A-chain homodimer, or an A/B heterodimer. There is also a 24kD variant of PDGF-BB that is retained in the endoplasmatic reticulum, rather than being secreted [92]. The different sub-types have partially different effects. The BB homodimer has been shown to be the most potent version and the PDGF-B gene will be the focus of this thesis.

There are two related types of PDGF transmembrane receptors, α and β, that are characterised by extracellular immunoglobulin-like ligand
binding domains and intracellular tyrosine kinase domains. The α-receptors and β-receptors can form homodimers or a heterodimer that have different binding affinities for the different PDGF ligands [94]. Binding of the PDGF ligand to the receptor induces autophosphorylation of the intracellular tyrosine domains, as well as phosphorylation of other substrates associated with the receptor. An intracellular signalling cascade involving PI3-K, GAP, PLC and src, then leads to the induction of more than 80 early response genes including transcription factors such as c-myc, fos and jun. These primary response genes in their turn induce downstream expression of transcription factors, structural proteins and cytokines, reviewed in [92].

The PDGF-B gene
The single-copy PDGF-B gene is located on chromosome 22 and spans approximately 22 kb [95]. It consists of seven exons and encodes a principal 3.5 kb mRNA, most of which is untranslated, except for a 723 bp long open reading frame. The 5′ untranslated region is GC-rich and contains a number of short open reading frames (SORFs) that confer inhibitory effects on translation [96]. A minor 2.8 kb transcript has also been described that lacks first exon sequences and probably originates from an alternative promoter located in the first exon [97]. The translation inhibitory signals contained in the 5′ UTR are lacking from this transcript, which is also the case for the transforming v-sis gene [98]. The functional differences between these alternative transcripts are not known and they encode the same protein. The 3′ UTR contains an AU rich sequence that has, however, been shown not to affect the stability of the transcript [99]. A number of investigations show other alternative transcripts of PDGF-B, although it remains unclear where these transcripts originate from [100, 101].

The 3.5 kb main transcript originates from a promoter with a consensus TATA-box 30 bp upstream of the transcription start site [102]. The promoter also contains two consensus Inr sequences, located at the Cap-site and immediately upstream of the TATA-box, respectively [103]. The sequence of the promoter region including 100 bp upstream of the start-site is 98% conserved between human and mouse. This minimal promoter has been shown to be sufficient for basal expression of PDGF-B in a number of cell types and contains a number of binding sites for constitutive and inducible transcription factors including AP-like and ETS-like binding sites [104]. A sis proximal element (SPE) located at –58 to –39 has been shown to bind a number of different factors in different cell-types [105]. In bovine endothelial cells the SPE has been shown to bind a thrombin inducible factor that mediates induction by the potent expression stimulator thrombin [106]. In human endothelial cells, SP-1 and the early growth response gene
Egr-1 factor compete for sequences within the SPE [107] and in U2-OS osteosarcoma cells, Sp-1 and two uncharacterised factors bind the SPE [105]. Regulatory regions have also been characterised further upstream in the PDGF-B promoter, including an activating element at −292 to −278 [108] and an ERK dependent endothelial specific repressor at −227 to −221 [109]. In addition to thrombin, PDGF-B transcriptional activity has been shown to be induced by various other stimuli, such as cyclic strain [110], shear stress via an NFκB element [111] and TPA during megakaryocyte differentiation in K562 cells [112]. PDGF-B expression has also been shown to be induced by hypoxia in endothelial cells [113], hepatocellular carcinoma cells [114] and megakaryocytes [115], although the mechanism and possible regulatory regions have not been defined. Long range regulatory mechanisms of PDGF-B will be further discussed below. The intricate and cell-type specific regulatory mechanisms controlling PDGF-B transcription suggests that a strict control of expression is required.

Physiological roles for PDGF-B
Since the initial characterisation of PDGF-B as a mitogen for fibroblasts and cells of mesenchymal origin, the picture has been proven to be more complex. PDGF-B is a multifunctional factor that is expressed by a very large number of cell-types. The two receptors for PDGF have also been shown to be expressed by many different cell-types and sometimes by the same cells that express the PDGF ligands, indicating a possible autocrine mode of action [92].

During embryonic development, PDGF-B has been shown to be important for the proper development of a number of organs such as testicles [116], central nervous system [92], the vascular system [117] and the placenta, which will be further discussed below. PDGF-B knockout mice die perinatally due to fatal bleeding and deformed kidneys, suggesting a developmental role for PDGF-B during vasculogenesis and kidney development [118]. In the adult organism, PDGF-B is important during wound healing [119].

PDGF-B and cancer
Turning to the pathological consequences, aberrant PDGF-B expression has been implicated in atherosclerosis [119], diverse fibrotic conditions [92] and in transformation and cancer. As mentioned earlier, the identification of PDGF-B as the cellular homologue of a virally transduced oncogene put the possible involvement of this factor in cancer into focus. PDGF-B has been shown experimentally to be able to induce transformation of receptor positive cells in vitro [120, 121] and to induce tumours following injections
of c-sis-carrying viruses into mice [122]. It is also upregulated in a wide range of tumours compared to the corresponding normal tissue and is expressed in many transformed cell-lines, further strengthening the link to neoplasia [92]. The co-expression of PDGF-B and its receptor in a tumour does not, however, prove a role for autocrine stimulation. It is possible in some cases that the potential autocrine stimulation is a causative agent during carcinogenesis, whereas it may only support the already established tumour in other cases. It has been argued that autocrine PDGF stimulation is critical in the development of certain gliomas [123, 124] and sarcomas [125, 126], although an autocrine interaction is difficult to formally prove.

PDGF-B has also been shown to contribute to tumour formation by paracrine stimulation of stromal cells and by its role in angiogenesis. In several tumour types, PDGF-B is expressed by the tumour cells and its cognate receptor in stromal fibroblasts [127, 128]. An elegant study using human melanoma cells injected in mice, illustrated the importance of PDGF-B in stroma recruitment [129]. Melanoma cells lacking PDGF-B expression contained no connective tissue stroma, few blood vessels and large necrotic areas. When the melanoma cells were manipulated to express PDGF-B, the tumours displayed a rich stroma containing numerous blood vessels and showed no signs of necrosis. Apart from supporting blood vessels via stroma recruitment, PDGF-B can also exert a direct effect on tumour angiogenesis. Co-expression of PDGF-B and the β-receptor by endothelial cells in capillaries of gliomas suggests an autocrine role in tumour vascularisation [124]. The expression of both ligand and receptor in endothelial cells indicates an angiogenic potential of PDGF-B [130], which has, however, been shown to be less potent compared to vascular endothelial growth factor, VEGF. Comparison of in vitro and in vivo experiments show that although PDGF-B is angiogenic, the main function in normal angiogenesis is the recruitment of smooth muscle cells and pericytes during vessel maturation [131]. It could be hypothesised, however, that PDGF-B could have a wider role during tumour angiogenesis due to micro-environmental factors like tumour hypoxia, which will be discussed further in a following section. The placenta is a structure that resembles tumours in the sense that it is rapidly proliferating, invasive and requires extensive vascularisation. PDGF-B has been shown to have multiple roles in the biology of this organ.

**Regulation of PDGF-B in placental cells**
In addition to displaying properties similar to tumours, the placenta is an interesting model for the study of the biological role of growth factors. PDGF-B may participate in placental angiogenesis by forming auto-
stimulatory loops that will induce proliferation of micro capillary endothelial cells, followed by paracrine stimulation of fibroblasts and smooth muscle cells in the formation of larger vessels [131]. In placental development, PDGF-B has been shown to regulate the proliferation of cytotrophoblast cells, probably via an autocrine loop [132]. Complete hydatidiform mole is a benign placental disease characterised by lack of the maternal genome. A small fraction of these progress to invasive moles and finally choriocarcinoma. The PDGF-β receptor expression is increased in mole compared to normal trophoblasts and the transition from benign mole to malignant choriocarcinoma is correlated with a 10 to 20-fold activation of the PDGF-B gene, indicating a possible auto-stimulatory role for PDGF-B in progression to malignancy [133].

Given the numerous roles for PDGF-B during placental development and the over-expression observed in choriocarcinoma, it was of importance to characterise the mechanisms of transcriptional regulation in placental cells. Detailed analyses have indicated specific transcriptional regulatory elements in placental cytotrophoblasts and the choriocarcinoma cell-line JEG-3. In cytotrophoblasts, both the main 3.5 kb transcript and the 2.8 kb transcript are expressed [97]. In cultured JEG-3 cells, however, only the smaller transcript that lacks exon 1 derived sequences is expressed. This has been suggested to result from excessive methylation of the promoter and/or first exon, a phenomenon often reported as a consequence of long-term cell culture.

DNase hypersensitivity studies performed in order to identify potential cell-type specific regulatory elements indicated two hypersensitive sites that were common for JEG-3 cells and cytotrophoblasts, located in the first intron. These hypersensitive sites were shown to be specific for placental cells and could not be detected using osteosarcoma cells or non-expressing fibroblasts. Transient transfections of reporter gene constructs carrying a basal PDGF-B promoter plus segments of the first intron performed in JEG-3 cells have shown that the 8 kb first intron enhanced the basal promoter 26-fold. When tested with the viral SV40 promoter, the 8 kb intron fragment showed only a 2-fold activation, disqualifying it as a “classical enhancer”, since it was not capable of significantly activating heterologous promoters [97]. Furthermore, its enhancer function was shown to be orientation-dependent, unless stably integrated into chromatin. A 2 kb intronic sub-fragment (termed throughout this thesis as XX) showed 10-fold activation of the basal promoter, retained the promoter specificity and was only able to activate when in positive orientation with respect to the promoter [97]. It appeared that the function of this non-classical enhancer was dependent on specific interaction with its own promoter; the first such element to be
HYPOXIA

It is clear that a fundamental aspect of gene regulation in general and the regulation of growth factor encoding genes in particular, is the effect of external stimuli such as concentration of essential nutrients and other circulating factors. These extracellular stimuli are detected by the cells in various ways and the signals ultimately mediate changes in gene expression and downstream effects on cell behaviour. One essential extracellular signal is the availability of oxygen. Virtually all eukaryotic cells depend on oxygen to generate metabolic energy. All multi-cellular organisms have developed intricate systems to assure a sufficient cellular level of oxygen. In mammals, all cells have to be located in the vicinity (100µm) of capillaries in order to receive enough oxygen. Nevertheless, there are a number of instances when the amount of oxygen becomes limiting and cells are subjected to low oxygen tension, a condition known as hypoxia.

Hypoxia occurs when the metabolic demands of a cell are not met by the supply of oxygen via the blood, like during early development [134], in muscle during heavy exercise, pathological states associated with reduced blood flow and in solid tumours, which will be discussed in more detail below. Cells and organisms have consequently evolved mechanisms to detect and respond to hypoxia. These responses result in a metabolic switch to a glycolytic pathway of energy production and ultimately to the restoration of oxygen supply via angiogenesis and increased erythrocyte production, by induction of a subset of genes [135].

The Hypoxia Inducible Factor 1 pathway

A majority of genes induced by hypoxia are controlled via the hypoxia inducible factor 1 (HIF-1), which was originally shown to control the hypoxic induction of the erythropoietin gene, thereby increasing erythrocyte production [136]. Subsequently, HIF-1 was shown to induce a large number of genes involved in controlling angiogenesis, glycolytic metabolism, and apoptosis/cell survival. HIF-1 is a heterodimeric transcription factor consisting of the constitutive subunit HIF-1β/ARNT and the hypoxia inducible HIF-1α reviewed in [135]. The exact mechanism of cellular oxygen sensing has not been defined, but this signal is somehow transduced to HIF-1α and regulates protein stability, nuclear translocation and cofactor recruitment in response to hypoxia. ARNT, however, is constitutively expressed and also dimerises with other partners in response to xenobiotics.
HIF-1α is a member of the helix-loop-helix, PAS-domain class of transcription factors that also includes HIF-2α/EPAS, HIF-3α and ARNT. HIF-2α was first thought to be specific for endothelial cells, although recent results suggest a wider expression of this factor [138]. The functional overlap between HIF-1α and HIF-2α still remains to be determined. HIF-1 has been shown to be primarily controlled by a hypoxia-mediated increase in protein stability. Furthermore, HIF-1 has also been shown to be induced by other stimuli associated with cell proliferation, such as PDGF-B [139], IGF-II [140] and the products of the ras and src oncogenes [141].

HIF-1 induces downstream genes by binding to a specific sequence (RCGTG) in the regulatory regions of these genes. Transcription is then activated partly via recruitment of co-activators with histone acetylase activity [78, 142]. In a few cases, factors such as the transcription factors AP-1 [143, 144] and Egr-1 [145] have been shown to mediate hypoxic induction of downstream genes, independent of the HIF-1 pathway. Hypoxia has also been shown to activate some genes by increasing the mRNA stability [146].

The importance of an accurate response to hypoxia during development is demonstrated by the fact that HIF-1α deficient mice die before birth and display severe developmental abnormalities, including defects in cardiovascular development [147-149]. These observed phenotypes largely overlap with those displayed by mice deficient for ARNT [150], or vascular endothelial growth factor (VEGF) [151], which is induced by HIF-1 and is a key regulator of angiogenesis. The two published reports on HIF-2α knockouts, however, show contradictory results. In one case, the absence of post vascular remodelling suggesting an important role in developmental vascularisation [152]. In the other case, however, there was no effect on vascularisation, but a defect in catecholamine synthesis, suggesting a role for HIF-2α in foetal oxygen sensing by extra-adrenal paraganglia [153].

Given the roles for PDGF-B in processes such as wound healing, angiogenesis and cancer, the mechanisms and pathways involved in PDGF-B regulation by the level of oxygen would be important to examine. The hypoxic regulation of PDGF-B is examined in paper IV of this thesis.

**Tumour hypoxia**

One condition during which hypoxia and gene regulation by hypoxia is of great importance is in the development of solid tumours. A tumour cannot grow beyond the size of 1 mm³, without acquiring a blood supply from capillaries. Since tumour cells proliferate rapidly, there are always zones of tumour cells that are subjected to hypoxia. Tumour hypoxia has been shown to be the trigger for recruitment of host vessels and capillary growth within
the tumour, via induction of angiogenic growth factor genes such as VEGF and PDGF-B [154]. The tumour vessels differ in a number of aspects from normal host vessels, displaying irregular flow, blind ends and lack of smooth muscle cells. Although both normal and tumour vessels primarily consist of host derived endothelial cells, they display distinct gene expression profiles [155].

Apart from inducing angiogenesis and thereby promoting tumour survival, tumour hypoxia also effects treatment and metastatic potential, reviewed in [156]. Radiotherapy is dependent on the intracellular production of reactive oxygen species, resulting in the resistance of hypoxic tumour cells to treatment. These cells also resist chemotherapy, since this type of treatment functions on rapidly proliferating cells. Hypoxic cells have a low proliferation rate and reside at a distance from vessels, resulting in that they receive low concentrations of the therapeutic agent diffusing from the vessels.

Figure 3. Effects of hypoxia on tumours

Limited tumour blood supply also results in a depletion of nutrients such as glucose. Low glucose, hypoglycaemia, like hypoxia, results in a switch to glycolysis and increased angiogenesis. Hypoglycaemia, has been shown to induce a subset of genes that partly overlaps with those induced by
hypoxia [157], although it is not clear to what extent this is dependent on HIF-1 [158]. HIF-1 has been shown to be over-expressed in a large proportion of tumours, which correlates with poor prognosis [141]. Experiments with xenogenic tumours in mice show that HIF-1 is required for angiogenesis and tumour survival, although some reports indicate that lack of HIF-1 actually results in larger tumours [149, 159].

**Neuroblastoma**

Neuroblastoma constitutes an interesting tumour type with regard to studying tumour hypoxia. It is a malignant paediatric tumour of the developing sympathetic nervous system and one of the most common extra-cranial tumours of early childhood with an incidence of 9/1 000 000 children aged under 15 [160]. Clinically, it is a heterogeneous disease ranging from highly malignant disease to spontaneous regression. Neuroblastoma occurs at an early age and is thought to originate from primitive neuroblastic cells that fail to differentiate into the ganglionic neurones or endocrine chromaffin cells that constitute the sympathetic nervous system [161]. It is interesting to note that these cells have been shown to be involved in the regulation of oxygen homeostasis in the embryo and foetus, possibly involving HIF-2α/EPAS [153]. Neuroblastoma is also interesting since it originates from undifferentiated foetal cells and the tumour has been shown to undergo differentiation along different pathways. The role of hypoxia in neuroblastoma development is explored in paper V of this thesis.
AIMS OF THE PRESENT INVESTIGATION

The aims of this work were to further characterise the interplay between *cis*-acting sequences, trans-acting factors, chromatin structure and environmental cues in the control of gene expression alterations that are important during normal development and in disease, such as in cancer. Specifically these studies were concentrated on answering the following questions:

- What are the regulatory elements involved in the specific regulation of the PDGF-B gene in tumour cells of placental origin?
- Are chromatin modifying activities important in the regulation of the PDGF-B gene?
- How does hypoxia affect the regulation of the PDGF-B gene?
- Is there a role for hypoxia in terms of gene regulation and tumour differentiation in neuroblastoma?
RESULTS AND DISCUSSION

A specific sequence element in the PDGF-B promoter is required for the interaction with its intronic enhancer (paper I).

To further characterise the non-classical behaviour of the previously described PDGF-B intronic enhancer in choriocarcinoma derived JEG-3 cells [97], we extended the analysis to include another cell types. Initial screening indicated that the intronic enhancer was functioning in the ZR-75 breast carcinoma cell line. Reporter gene analysis indicated that distinct properties of the intronic enhancer were cell type-specific. The 8 kb intron and 2 kb XX sub-fragment induced transcription of the main PDGF-B promoter, approximately 26-fold and 10 fold respectively, in both JEG-3 and ZR-75 cells. The level of induction was very similar, although the activity of the basal promoter was around 50-fold higher in ZR-75 cells, suggesting that the enhancer can synergise with the promoter and cause activation irrespective of basal promoter strength. In terms of the orientation dependence and promoter specificity described for the intronic enhancer in JEG-3 cells, the picture for the ZR-75 cells was shown to be somewhat different. While the 2 kb intronic sub-fragment was not functional in the negative orientation and could not activate the heterologous SV40 viral promoter in JEG-3 cells, the same experiment performed with ZR-75 cells showed a 50% reduction of enhancer activity using the negative orientation and a 50% reduction of enhancement, down to 5-fold, with the SV40 promoter. This result emphasised the cell-type specific differences between the enhancer/promoter interactions and indicated that distinct regions of the enhancer fragment were probably active in the two cell-types, although the degree of enhancement was identical.

In order to elucidate whether a specific sequence element in the PDGF-B promoter was responsible for the specific interaction with the intronic enhancer, a series of 18 bp replacement mutants covering the promoter were generated. These promoter mutants were then tested for basal activity and their potential to be activated by the enhancer, using transient transfections of reporter gene constructs. A specific sequence, termed the EDC (Enhancer Dependent cis co-activator), was subsequently identified. The EDC consists of 22 bp flanking, but not including the TATA-box and contains an Inr consensus sequence. Mutation of the EDC sequence did not affect basal promoter activity, but eliminated the 10-fold activation by the XX intronic enhancer fragment. These experiments also showed that the TATA-box is not required for basal or enhancer activated transcription from the PDGF-B promoter, suggesting that a second initiator motif located
at the CAP site could initiate accurate transcription. The TATA proximal Inr element, on the other hand, was required for the interaction with the enhancer, revealing a novel type of function of the Inr type of elements. The requirement for the EDC was shown to be cell type-specific, since the intronic enhancer activated both wild-type and EDC-mutated promoters 10-fold in the breast cancer cell-line ZR-75, indicating distinct mechanisms of enhancer activation in these two cell types. Taken together, these data indicate that the intronic enhancer behaves in a quasi-classical manner in ZR-75 cells, but in JEG-3, the enhancer/promoter interaction is highly specific and dependent on a novel sequence element in the promoter.

The characterisation of this cell-type specific, non-classical enhancer/promoter system prompted further investigations into the function of the enhancer itself, both in terms of cis-elements involved and in the context of a possible enhancer mechanism.

**Characterisation of functional elements in the intronic enhancer and identification of a specificity element (paper II)**

After having characterised the promoter elements responsible for the specific interaction with the enhancer, the obvious thing to do was to take a closer look at the enhancer. Paper II describes the functional dissection of the 2 kb intronic enhancer in terms of elements responsible for both promoter specificity and transactivation. The 2 kb enhancer was analysed by transient transfections of reporter constructs containing sub-regions of the enhancer linked to a wild-type, or EDC-mutated promoter. Using this approach, a more classical type of enhancer element was isolated. This element, located centrally in the 2 kb fragment, worked irrespective of orientation, could activate a heterologous SV40 promoter and was independent of the EDC sequence in the promoter. Furthermore, it had synergistic effect when placed in tandem in front of the promoter, causing 65-fold activation, which is also a hallmark of classical enhancer function. When this enhancer fragment was spaced away from the promoter, however, it failed to activate the PDGF-B promoter, a fact that does not match with the expectation of a classical enhancer to work irrespective of distance to the target promoter. In this sense therefore, the isolated fragment behaved more like an upstream promoter element.

Next, the 5' region of the 2 kb enhancer was analysed. This region was capable of activating both the wild-type and EDC-mutated promoter, but only from a proximal position. The addition of 27 extra base pairs of 3´sequence, however, made this fragment capable of activating only the wild-type promoter. This observation indicated that the 27 bp fragment is responsible for the enhancer specificity. In order to test this, a 2 kb enhancer
was constructed with the 27 bp region mutated: this had no effect on the lack of activation of the EDC-mutated promoter, whereas the 10-fold activation of the wild-type promoter was completely neutralised, indicating that the 27 basepairs are necessary for the specific enhancer-activation. This 27 bp region was subsequently termed EDC specificity element (EDCse). It was further shown that the EDCse had no intrinsic activation potential. The enhancer consists, therefore of two regions with activating potential, one central and one in the 5′ region of the 2 kb fragment and in addition, a specific element that confers the promoter specificity is also required.

It remains to be explained why the activating regions failed to function unless located in a promoter-proximal position. The EDCse is obviously required to bring distally bound activators to the promoter and the two activators have to synergise with both each other and the EDCse in order to function distally. Removal of the EDCse had no effect on the enhancer activation of wild-type or mutated promoters in the ZR-75 cell-line, reflecting the EDC-independent mechanism of activation in these cells. In conclusion, both the EDC and the EDCse are required for enhancer activation in the JEG-3 cells. A hypothetical factor bound to the EDCse could be envisaged to interact, directly or via co-factors, with a factor bound to the EDC-element, thereby enabling the distal enhancer-bound activators to interact with the transcriptional machinery.

The analysis was then further extended to the level of DNA/protein interactions. In an electrophoretic mobility shift assay (EMSA), it was shown that the 27 bp EDCse specifically binds a protein present in a JEG-3 extract. Using ZR-75 extract, a protein complex of different size was shown to bind, correlating with the different functions of the EDCse in JEG-3 and ZR-75 cells. Database searches performed to identify putative factors binding to the EDCse did not result in any identification of likely candidates, except members of the GATA-family of transcriptional activators. The EDCse contains a consensus GATA-binding sequence, but mutation of this did not affect the results of the EMSA. Furthermore, when a footprint assay using JEG-3 extracts was performed, protein binding was mapped to a region outside of the GATA-consensus sequence. Two footprints were detected, one located 5′ in the 27 bp fragment, and a weaker one located further 3′. It should be noted that database searches of putative DNA-binding factors are dependent on that the factor has been previously characterised and even if a match is found, this is far from proof of actual binding or functionality in vivo. It could be hypothesised that these cell-type specific interactions would utilise a protein factor that is also very specific.

It is valid to ask why such a complex regulatory mechanism has evolved in choriocarcinoma cells. Previous reports of specific
enhancer/promoter interactions have mainly been described in regions where competition for enhancers between a number of clustered promoters could occur. Two putative promoters inside the actual PDGF-B gene have been described, one of which is assumed to be located in the central enhancer region [100]. This promoter activity is, however, extremely weak and our current study shows that the role of this region is mainly as an enhancer. The other alternative promoter, located at the end of exon 1, has previously been shown not to be activated by the intronic enhancer [97]. The function of the EDC/EDCse system could then be to prevent this promoter from being activated by the intronic enhancer. The function of this putative promoter remains to be characterised in more detail. A third proximal promoter is the main PDGF-B promoter itself, which we have shown to function bidirectionally (unpublished data). Enhancer activation of the reverse promoter activity, however, seems to be independent of the EDC-element. Regardless of the biological function, the specific regulatory system described in this thesis is still interesting as a model system for an intricate enhancer/promoter specific mechanism of strict gene regulation and was the first such system to be described and characterised in detail.

The effect of histone acetylation status on the function of the PDGF-B enhancer/promoter system (paper III)

After having analysed the cis-elements and to some extent, the protein factors responsible for the specific regulation of PDGF-B in JEG-3 cells, the focus was directed towards chromatin aspects of this system. One of the most widely recognised correlations between chromatin and transcription is the effect of histone acetylation status. Activated transcription has been correlated with hyper-acetylated histones, while repression and silent genes have been correlated with hypo-acetylated histones [63]. Both the general transcription machinery and sequence specific DNA-binding activators/repressors have been demonstrated to associate with factors harbouring acetylase or deacetylase activity. In this context, it was interesting to extend the analysis of the cell-type specific PDGF-B transcriptional regulation.

Paper III analyses the possible effects of histone acetylation status on the transcriptional regulation of PDGF-B. Initially it was noted that the histone deacetylase inhibitor Trichostatin A (TSA) had different effects on transcription from the main PDGF-B promoter, depending on its basal activity in different cell-types. A number of cell-types were transiently transfected with the PDGF-B promoter coupled to a reporter-gene. It was shown that TSA strongly activated the promoter in JEG-3 cells, where the basal level of expression is low. In contrast, TSA addition had little effect in
cell-lines where the promoter displayed a higher basal activity, such as ZR-75, hepatocellular carcinoma Hep3B and the rhabdomyosarcoma cell-line RD. The low basal promoter activity could be induced 10-fold by addition of TSA in JEG-3 cells. This indicates that the low basal activity of the PDGF-B promoter in JEG-3 cells could be explained by assuming that the promoter is in a default hypo-acetylated state.

It was then observed that a reporter-gene construct including the 2 kb intronic enhancer was activated only 2-fold by TSA. The level of enhancer activation was very similar to the level of TSA activation seen with the basal promoter. This could be interpreted as a common activation mechanism between the enhancer activation and the activation by TSA. It has been shown earlier that when specific promoters are activated by enhancers via recruitment of histone acetylase activity, they are not further induced by the addition of TSA [162]. It is also possible that the lack of potent TSA activation seen with the enhancer is due to an element in the enhancer that could block the TSA effect. To test this, the effect of TSA on a construct containing the 2 kb enhancer and the EDC-mutated PDGF-B promoter, which the enhancer could not activate, was analysed. Addition of TSA activated this construct to almost the same degree as the basal promoter alone, indicating that the presence of the enhancer per se did not interfere with TSA activation.

It could be argued that analysis of the relation between chromatin structure and transcriptional activity cannot be accurately performed using transiently transfected plasmids. In order to order meet this argument, the nucleosome structure of the transfected plasmids was analysed by MNase digestions and Southern blots. These experiments indicated that nucleosomes were present on the transfected plasmids, which has also been shown in earlier studies [163]. Furthermore, the reporter-gene constructs were integrated in the genome by co-transfection with a neomycin resistance gene plasmid and the stable transfectants were then assayed for activation by TSA. The results matched those obtained using transient transfections, showing a strong activation of the basal promoter and a very modest activation of the enhancer-activated promoter, further strengthening the possibility of a direct effect on nucleosome structure. These data suggest that the basal promoter is associated with hypo-acetylated histones by default and the effect of TSA occurs via altering the balance between acetylase/deacetylase activity, thereby activating transcription. It could also be hypothesised that addition of TSA activates another gene, which in its turn has an effect on PDGF-B expression, possibly by altering chromatin structure. This has been shown to be the case for the cell-cycle regulatory protein p21 gene, which is activated by TSA via an Sp1 site in its promoter.
In order to find out if the TSA effect could be tied down to a specific promoter element, the replacement mutants used to identify the EDC were tested for TSA inducibility. None of the mutants had any effect on the level of activation by TSA, indicating that the PDGF-B promoter does not contain a “TSA-responsive element”. Rather, the effect of TSA, and possibly of the enhancer, could be to modulate the balance between hypo- and hyper-acetylated histones. It is difficult, however, to directly assess activator function in vivo.

Next, the effect of TSA on the endogenous expression of PDGF-B in JEG-3 cells was analysed by RNase protection assay. Modulation of acetylation status has earlier been shown to reactivate expression of some genes silenced as a result of in vitro culture [165]. Addition of TSA was not sufficient to activate the normally repressed main PDGF-B promoter, since no transcript encompassing exon 1 sequences could be detected. This fact was speculated to be due to excessive methylation, which may be dominant over acetylation status in terms of repression. Treatment of the JEG-3 cells with the demethylating agent 5-azacytidine, however, still did not enable the PDGF-B promoter to be activated by TSA, possibly due to residual methylation of the promoter and/or first exon. Southern blotting of DNA digested with a methylation sensitive restriction enzyme indicated that the promoter region is heavily methylated, which could be at least partially overcome by the addition of 5-azacytidine. TSA alone had no effect on the methylation status, whereas combined treatment with 5-azacytidine and TSA resulted in almost complete demethylation, indicating a possible role of TSA in modulating methylation status. Methylation analysis using restriction enzymes however, only probes a subset of potentially methylated CpGs and does not give a full picture of the methylation status.

When the effect of TSA on the smaller PDGF-B transcript, lacking exon 1 derived sequences was analysed, however, the picture was different. The smaller transcript, possibly originating from a promoter located in exon 1 [101], was shown to be moderately, but consistently, repressed by TSA. This is consistent with the finding that this putative promoter is not activated by the intronic enhancer, indicating that the two promoters are regulated by separate mechanisms [97].

The effect of chronic hypoxia on PDGF-B expression (paper IV)
Having analysed the intricate network of cis-elements and the effect of chromatin on the regulation of PDGF-B, the focus of our studies were concentrated on how external environmental cues could regulate PDGF-B expression. Hypoxia has been shown to induce PDGF-B expression in endothelial cells [113] as well as in the hepatocellular carcinoma cell lines
Hep3B and HepG2 [114], although the precise mechanism was not elucidated. Given the numerous roles for PDGF-B in processes involving hypoxic states, such as angiogenesis and tumourigenesis [92] we set out to further characterise the effect of hypoxia on PDGF-B gene expression. As model systems, Hep3B cells and the transitional cell bladder carcinoma cell line T24, which has been shown to express high levels of the main PDGF-B transcript [99], were chosen. Furthermore, PDGF-B expression has been shown to be correlated with micro-vascular density in bladder carcinoma [166].

Using RNAse protection assays, the levels of PDGF-B mRNA in T24 and Hep3B cells that had been exposed to 0.1% oxygen for different periods of time were analysed. The results showed that in T24 cells, PDGF-B expression was induced after 16-24 hours of hypoxic exposure. More surprisingly, the mRNA levels were repressed significantly below basal level after long-term hypoxic exposure (96 hours). This biphasic response to hypoxia was shown to be cell type specific, since PDGF-B expression was also induced by hypoxia in Hep3B cells, but remained elevated after long-term hypoxic exposure. The biphasic response to hypoxia compared with other hypoxia induced genes was then examined. Using RNase protection assays, the effect of hypoxia on the expression of the genes for vascular endothelial growth factor (VEGF) and heme oxygenase 1 (HO-1), which have previously been shown to be induced by hypoxia in various cell-types [167, 168], was tested. VEGF and HO-1 expression was indeed induced by hypoxia in T24 cells, but these genes were not repressed after long term hypoxia, suggesting this response to be specific for PDGF-B. It was also shown in this system that addition of cobalt chloride, which mimics hypoxia, did not induce PDGF-B, while inhibition of protein synthesis abrogated the hypoxic induction. Using actinomycin D treatment it was then shown that the induction of PDGF-B expression was not via a hypoxia-mediated increase in RNA stability. We next tested the effect of a set of possible mediators of the observed repression, such as carbon monoxide and cycloheximide that did not have any effect on the observed repression.

It was found, however, that the repression observed after long-term hypoxia could be abrogated by supplying the cells with excess glucose, indicating that the long term repression was a function of hypoglycaemia caused by the long term hypoxia. Hypoxia often results from a local reduction in blood flow, a state that will also limit the availability of essential nutrients such as glucose and result in hypoglycaemia. The cells adapt to these stimuli by inducing a subset of genes, including VEGF and genes encoding enzymes of the glycolytic pathway. It is not clear to what extent these genes are induced via the same mechanism by these two related
stimuli. In order to further analyse the effect of low glucose on the expression of the PDGF-B gene, RNase protection assays on RNA from T24 cells maintained in hypoxia or normoxia were performed. These studies showed that hypoglycaemia repressed PDGF-B mRNA below control levels and that the combined effect of hypoxia and hypoglycaemia led to an even stronger repression. This indicates that glucose levels are critical for PDGF-B expression and are dominant over the inducing effect of hypoxia. These results clearly show that in contrast to many other hypoxia induced genes, PDGF-B is repressed by hypoglycaemia, which also supports the idea that these stimuli do not operate via a common induction mechanism in all cases.

A majority of hypoxia induced genes have been shown to be regulated via the master regulatory transcription factor HIF-1. Direct induction via HIF-1 requires de novo protein synthesis, can be mimicked by certain metal ions and occurs via HIF-1 binding to specific sequence elements. It has not been determined if hypoxic induction of PDGF-B is mediated via HIF-1. A putative HIF-1 response element (HRE) was identified in the upstream region of the PDGF-B promoter. This putative HRE contained a consensus HIF-1 binding site and a consensus ancillary sequence that has been shown to be required for hypoxic induction of VEGF [169]. When a reporter gene construct with the PDGF-B promoter containing the putative HRE sequence was transfected into T24 or Hep3B cells, it could not, however, confer hypoxic inducibility. Electrophoretic mobility shift assays (EMSA) confirmed that the putative HRE did not bind any proteins induced under hypoxic conditions. These results indicated that the putative HRE was not sufficient to mediate the hypoxic induction observed for the endogenous PDGF-B gene. The question remains open as to whether other potential HRE sequences are required, or if the PDGF-B gene is induced by hypoxia via an HIF-1 independent pathway. It was, however, shown by reporter gene assays that a sequence encompassing the putative HRE element functioned as a cell-type specific activator of basal transcription in Hep3B cells. Other transcription factors, distinct from HIF-1, have also been shown to bind the same consensus sequence. In conclusion, we demonstrated that unlike any other known gene hypoxia and hypoglycaemia have antagonistic roles in the regulation of PDGF-B gene expression. The exact mode of hypoxic induction requires further study.
Hypoxia is involved in tumour differentiation in neuroblastoma (paper V)

It is clear that hypoxia indirectly promotes tumour survival by inducing genes that control angiogenesis. We were interested to find out whether hypoxia could also be involved in other features of tumour development. Neuroblastoma tumours are especially interesting in this sense, since they originate from primitive cells of the sympathetic nervous system (SNS) [161]. The SNS consists of ganglionic neurons, supportive glia cells and chromaffin cells with neuroendocrine function. The chromaffin cells are located in the adrenal gland, extra-adrenally in the paraganglia or within sympathetic ganglia as small intensely fluorescent (SIF) cells. In the developing embryo and early foetus, the extra-adrenal chromaffin cells control oxygen homeostasis by sensing blood levels of oxygen and releasing catecholamines, thereby regulating heart-rate and global haemodynamic response. This response has been indicated to be dependent on the hypoxia induced transcription factor HIF-2/EPAS [153].

In order to study the effect of hypoxia on neuroblastoma in terms of gene expression, differentiation and potential treatment regimes, a number of in vivo and in vitro studies were performed. Analysis of a large set of fixed tumour specimens (116 samples, representing different clinical stages) by in situ hybridisation and immunohistochemistry showed that the major form of differentiation in neuroblastoma was towards neuroendocrine chromaffin-like cells, rather than towards ganglionic neurons. These results were in contrast to earlier studies but it should be noted that those were based on far fewer tumour samples. Furthermore, the neuroendocrine chromaffin markers (IGF2 expression, chromogranin A staining, tyrosine hydroxylase activity, absence of GAP43 expression and morphological criteria) were shown to co-localise with regions of hypoxia, as judged by VEGF expression and distance from vessels as well as proximity to necrotic/apoptotic areas. Chromaffin-like differentiation was mainly a feature of stroma poor tumours, while cell differentiation along the ganglionic lineage was exclusively seen in the few tumours were stroma-rich. These observations indicated that hypoxia could be involved in the regional differentiation of neuroblastoma along a neuroendocrine pathway. Statistical analysis showed that the chromaffin like features observed were not correlated with clinical outcome, disease stage or site of origin of the examined tumours, although this was the most common pathway of differentiation.

In order to examine the generality and possible mechanism of the observed correlation between hypoxic zones and differentiation, the analysis was extended to xenogenic tumours. Cells from six different neuroblastoma cell lines were injected sub-cutaneously into nude mice and the resulting
tumours were analysed for expression of specific markers for both chromaffin and tumour hypoxia. The general trend in this experiment was similar to the results observed in clinical tumours: a co-localisation of chromaffin differentiation and hypoxic markers. These results also correlated well with foetal control samples, where expression of IGF2, chromogranin A and VEGF were specific for extra-adrenal chromaffin cells and expression of GAP43 was specific for ganglionic neurons.

Experiments with neuroblastoma cells in culture showed that the increased expression of IGF2 and VEGF specifically resulted from hypoxia, rather than a depletion of serum or glucose. The expression of HIF-2/EPAS in foetal control samples, as well as in clinical and experimental neuroblastoma tumours was then analysed. EPAS-1 expression was shown to be generally confined to extra adrenal chromaffin cells in the foetal samples and chromaffin differentiated regions of the tumours, suggesting a possible role for EPAS.

In summary, this study shows that the major pathway of differentiation in neuroblastoma is actually neuroendocrine in nature and may be mediated by tumour hypoxia. To our knowledge, this is the first data that suggest a role for hypoxia in tumour differentiation, in addition to the well characterised effects on angiogenesis, metastatic potential and resistance to treatment. These results also suggest a link between the natural function of paraganglionic neuroendocrine cells in controlling foetal oxygen homeostasis and the observed hypoxia-dependent differentiation. It remains to be determined to what degree the correlation between hypoxia and differentiation is causal and if so, whether the molecular mechanism involves IGF2 or EPAS induction.
Concluding remarks
The work contained in this thesis illustrates the complex, multiple level components that are involved in eukaryotic gene regulation. The study of one single gene (PDGF-B) has demonstrated the important contribution of genetic, epigenetic and physiological signalling mechanisms.

Initially, it was shown that cell-type specific promoter/enhancer interactions are involved in the regulation of the PDGF-B gene. The detailed characterisation revealed novel types of promoter and enhancer elements, which require specific DNA-binding factors apart from the general transcription complex in order to communicate with each other. This is the first promoter-specific enhancer system to be characterised in such molecular detail.

Chromatin modifying activities were subsequently shown to be involved in the function of the cis-elements described above. Inhibition of histone deacetylase activity induced the PDGF-B promoter in a manner which was inversely correlated with its basal activity in different cell-types. Moreover, in cells supporting the promoter-specific enhancer system, the promoter was refractory to HDAC inhibition when driven by the enhancer. These results indicate that the default acetylation status is cell type specific and that histone acetylation may be involved in the action of the promoter-specific enhancer.

It was then shown that hypoxia and hypoglycaemia have antagonistic effects on the expression of PDGF-B. This was a novel finding, since for the other genes that are known to be regulated by hypoxia and hypoglycaemia, these stimuli have been shown to have agonistic effects on expression. These results also re-emphasise the need to consider the potential effects of glucose metabolism in hypoxia studies.

Finally, hypoxia was indicated to be involved in the regional differentiation of neuroblastoma tumours. This is the first report of the involvement of hypoxia in tumour differentiation and may reflect the parasympathetic origin of this tumour type.

Although the individual studies have concentrated upon very specific issues, together they have provided novel insights into several areas, which are of wider scientific significance.
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