NEUTROPHIL ELASTASE GENE EXPRESSION IN HEALTH AND DISEASE

by

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ABSTRACT

Neutrophil elastase (NE) is a major protease carried by neutrophils and synthesized in the promyelocyte. A role of NE in the pathogenesis of emphysema was hypothesized over 25 years ago. Our understanding of the pathogenesis of this disease has remained obscure however, due to the lack of an accurate model for examining the causative role of proteases such as NE in the disease. The hypothesis is presented that over-expression of NE increases susceptibility to emphysema. Two transgenic models were proposed to **1**) develop a lung-specific NE transgene to examine the effects of NE over-expression in the lung, and **2**) determine the ability of the proximal human NE gene promoter region to drive expression of the gene in mice. The lung-specific promoter-driven NE transgene appeared to be toxic to the developing embryo, and the proximal promoter region of the human NE gene was insufficient to drive transgene expression, suggesting that additional regulatory elements are required to drive NE gene transcription in vivo.

To better understand NE gene regulation in vivo, the chromatin structure of the NE locus was examined. Seventeen DNase I hypersensitive sites (DHS) were discovered in an early myeloid cell line. Examination of non-expressing cell lines revealed that the chromatin organization at the locus is unique to NE-transcribing cells. To determine whether DHS flanking the NE gene are important in mediating NE transcription, reporter constructs carrying these sites were introduced transiently into an early myeloid cell line expressing NE. These studies confirmed a minimal functional promoter. The other DHS had no additional effect on transcription in this assay. To determine if these sites play a role in the presence of chromatin, stable transfections were performed. In this case, the minimal promoter was transcriptionally silent. Addition of a distal upstream hypersensitive site, DHS-9, increased expression by three-orders of magnitude. Since this effect was not observed in the transient studies, it can be concluded that DHS-9 is a chromatin-dependent enhancer of transcription. The requirement of DHS-9 in mediating high levels of transcription in the presence of chromatin thus identifies an important role of chromatin organization in NE gene expression in vivo.

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Part I. Development of Animal Models to Study the Role of Neutrophil Elastase in the Pathogenesis of Pulmonary Emphysema

Chapter 1: Introduction: Neutrophil Elastase and Emphysema

Since its discovery in 1968 (Janoff and Scherer, 1968), neutrophil elastase (NE)* has been extensively studied and implicated in several diseases, particularly those involving connective tissue damage accompanied by chronic or acute inflammation. Diseases in which NE has been studied as a putative pathogenic agent include pulmonary emphysema, adult respiratory distress syndrome (Donnelly et al. 1995), cystic fibrosis (Birrer et al. 1994; O'Connor et al. 1993), glomerulonephritis (Oda et al. 1997; Suzuki et al. 1993), rheumatoid arthritis (Janusz and Durham, 1997), and ischaemia-reperfusion injury of the myocardium (Haga et al. 1996; Tiefenbacher et al. 1997). In the past five years alone, there have been over 400 published studies on NE†, the majority of which examined NE as an agent in disease progression‡. Although a clearly defined role for NE in many of these diseases has yet to be substantiated, the existing evidence and simple attractiveness of NE as a pathogenic agent in these scenarios have fueled considerable efforts in both research and industry in the design and development of effective and safe inhibitors of NE for clinical use (Eriksson, 1991; Edwards and Bernstein, 1994).

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* Also, but less commonly, referred to as leukocyte elastase, PMN elastase, or medullasin

† Based on a search with MEDLINE, the bibliographic database of the National Library of Medicine, NIH, for publications from 1993 to 1998.

‡ Based on inspection of 60 arbitrary entries in MEDLINE between 1997-1998. 17% of articles were concerned with NE biology while the remainder were concerned with NE in disease or with development of NE inhibitors.

Part I of this thesis describes

1. the current understanding of the role of NE in the pathogenesis of emphysema,
2. deficiencies in our understanding of this concept,
3. the need to develop an improved experimental model to clarify the role of NE in acquired emphysema, and
4. the experimental approach we have taken in developing such a model.

Although the development of the anticipated experimental models of NE has remained elusive for reasons discussed and expanded upon in Part II, the experimental rationale and methods used are detailed in this section. The primary issue in Part I is why we and other investigators over the past two and a half decades have focused on NE rather than one of the numerous other endogenous and exogenous substances in the lung with the potential to contribute to the development of emphysema and how current controversies surrounding the hypothetical role of NE in the pathogenesis of emphysema may be resolved.

Emphysema

The clinical features, epidemiology, pathology, and pathophysiology of pulmonary emphysema have been extensively studied and are reviewed in a number of authoritative texts; the reader is referred to Thurlbeck and Wright's monograph of 1999 for a comprehensive overview of these subjects. Only the key features of emphysema pertinent to the present discussion are considered here.

Emphysema is a disease of the lungs in which the most significant clinical feature is chronic airflow obstruction; it is often classified together with chronic bronchitis, bronchiolitis, and chronic asthma

under the umbrella term 'chronic obstructive pulmonary disease' (COPD)*, providing a practical term for clinical diagnosis. In 1993, greater than 2 million individuals in the United States were estimated to have clinically apparent emphysema based upon self-reported conditions, with 17,990 deaths due to the disease that year (National Lung Health Education Program Executive Committee, 1998). Clinically apparent (moderate to severe) emphysema is almost always associated with tobacco use, and particularly cigarette smoking, and to a lesser extent with hereditary, homozygous deficiency of α_1-antitrypsin (α1AT) (Eriksson, 1996) and occupational exposure to dusts and fumes (Oxman et al. 1993). Familial differences in susceptibility to emphysema unrelated to α1AT deficiency and exclusive of environmental factors have also been documented (Redline et al. 1987; Sandford et al. 1997; Coultas et al. 1991), indicating that additional genetic factors are likely to be involved in determining an individual's susceptibility to development of the disease. Apart from hereditary deficiency of α1AT-deficiency however, such genetic factors have not yet been identified.

Subclinical emphysema is much more prevalent than clinically apparent emphysema. Numerous studies have shown that the majority of lungs from adult necropsies have emphysematous lesions (Thurlbeck and Wright, 1999). Airspace enlargement in the lung resembling emphysema also occurs with aging but can be distinguished from "true" emphysema, which by definition, is abnormal. Limits for normality of airspace enlargement have been proposed (Gillooly and Lamb, 1993). Whether airspace enlargement associated with aging is an intrinsic effect of the aging process or whether it follows the same mechanism as tobacco-smoke induced emphysema, i.e., through the effects of environmental pollution, is unknown.

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* Also, chronic obstructive lung disease, chronic obstructive airway disease, or chronic non-specific lung disease (COPD + asthma).

Pathology

Emphysema is defined at the anatomical level as "*a permanent, abnormal enlargement of any or all parts of the acinus, accompanied by destruction of respiratory tissue*" (Thurlbeck, 1995). Destruction of respiratory tissue has been defined by a committee of the National Heart Lung and Blood Institute as "nonuniformity in the pattern of respiratory airspace enlargement, so that the orderly appearance of the acinus is disturbed and may be lost" (National Heart, 1985). Microscopically, emphysematous lesions are characterized by enlarged and irregular airspaces and fenestrations in the alveolar wall, reduction of the alveolar capillary bed, and loss of alveolar attachments (Nagai et al. 1991b; Nagai and Thurlbeck, 1991a). Ultrastructural studies of collagen and elastin fibers reveal fibrosis of alveolar walls, as shown by thickened beds and bundles of collagen fibrils throughout the airspace walls (Finlay et al. 1996). Elastic tissue in the alveolar space normally exists in a regular arrangement, wrapping around the alveolar walls. In emphysematous lesions, these sheets of elastin are fractured and irregular. Biochemically, total elastin content appears to be decreased in emphysematous lesions (Cardoso et al. 1993), suggesting degradation of elastin as a cause of distorted elastic tissue in emphysema.

Several categories of emphysema are recognized, based upon the region of the acinus predominantly affected by the lesion (reviewed in Thurlbeck, 1976). These types of emphysema are in turn characteristic of different etiological circumstances. Centrilobular (centiacinar/ proximal acinar) emphysema is the most common type of emphysema associated with chronic airflow obstruction, being associated primarily with smoking-related disease. In centrilobular emphysema, the respiratory bronchioles of the acini are most extensively affected, and the alveolar ducts and alveoli are less involved or apparently normal. Centrilobular emphysema is found more frequently in the upper zones of the lung.

Panacinar (panlobular) emphysema involves the entire acinus uniformly, from the respiratory bronchioles to the alveoli. This type of emphysema is associated with hereditary α1AT-deficiency and is observed more frequently in the lower zones of the lung in severe disease. The relationships between the pathogenesis of centrilobular and panacinar emphysema are uncertain, but they can be observed in the

same lung in some cases. Severity of α1AT-deficiency associated emphysema is exacerbated by cigarette smoking (Eriksson, 1996), suggesting that the centrilobular and panacinar forms of emphysema may share a common pathogenetic route. α1AT-deficiency related emphysema is generally considered to provide some insight into the development of smoking-related emphysema, as a human "model" of the disease; however, it should be emphasized that the degree of similarity between the pathogenesis of cigarette smoke and α1AT-deficiency induced emphysema is only presumed as the pathogenesis of cigarette-smoke induced emphysema is unknown. Two additional forms of emphysema are observed, but their mechanisms of development appear to be distinct from the more clinically relevant centrilobular and panacinar forms of emphysema: Distal acinar (paraseptal) emphysema primarily affects the distal portion of the acinus (alveoli); it is rare and its clinico-pathologic associations vary. Irregular (paracicatricial) emphysema affects the acinus irregularly. It is usually not symptomatic on its own, and is believed to result from scarring, with which it is typically associated.

Pathogenesis

Although the relationship between enlargement of airspaces in emphysema and the accompanying destruction of respiratory tissue observed in these lesions is excluded from the formal definition of emphysema, it appears *prima facie* that destruction of alveolar walls in emphysema is a cause rather than effect of "abnormal permanent enlargement" of airspaces. This premise is common to prevalent theories regarding the pathogenesis of emphysema and is experimentally supported, as discussed below. The converse possibility, that permanent airspace enlargement resulting from over-distension, expiratory obstruction, or some other mechanical force, leads to destruction of the alveolar walls in emphysema, has been largely discredited (reviewed in Snider, 1992a). In emphysema, the entire acinus is affected: the alveolar epithelium, interstitium, extracellular matrix, basement membranes, and vascular endothelium are all disrupted. Any hypothesis regarding the pathogenesis of this disease must therefore be able to

account for the damage of both cellular and extracellular matrix components of pulmonary tissue and for lack of adequate repair.

Many exogenous and endogenous substances have the potential to contribute to the destruction of lung parenchyma seen in emphysematous lesions. The presence of numerous potentially destructive substances in cigarette smoke is a confounding factor in our understanding of smoke-induced emphysema. Cigarette smoke, the primary etiological agent of emphysema, contains over 4000 chemicals, a number of which could potentially elicit damage of pulmonary tissue directly or indirectly, through activation or recruitment of other damaging agents, inactivation of protective mechanisms, disruption of repair, or any combination of these. The lung itself is a complex organ, and hence it is not difficult to imagine how disruption of any of the many structures, substances, or processes in the normal lung might result in damage of the apparently fragile respiratory units. A key issue in advancing the current understanding of the development of emphysema is to understand whether the disease arises through the combined interaction between a horde of destructive substances and mechanisms, whether there is a simple, preponderant set of substances and sequence of events which leads to the disease, or perhaps several such pathways that differ between different individuals with this disease. The predominant theory used to describe the pathogenesis of emphysema is the 'protease-antiprotease hypothesis'. The protease-antiprotease hypothesis attributes the destruction and associated airspace enlargement in emphysema to the destructive capacity of excessive proteolytic activity in the lung stemming from an increased burden of proteases, a deficiency of the endogenous antiprotease "shield", or both (Fig 1.1). Discovery of the relationship between hereditary deficiency of α_1-antitrypsin and early onset emphysema promoted the idea of proteolytic involvement in emphysema, and together with recognition of tobacco smoke as the primary etiological agent of emphysema, remains the central observations in shaping our understanding of the mechanisms leading to the disease.

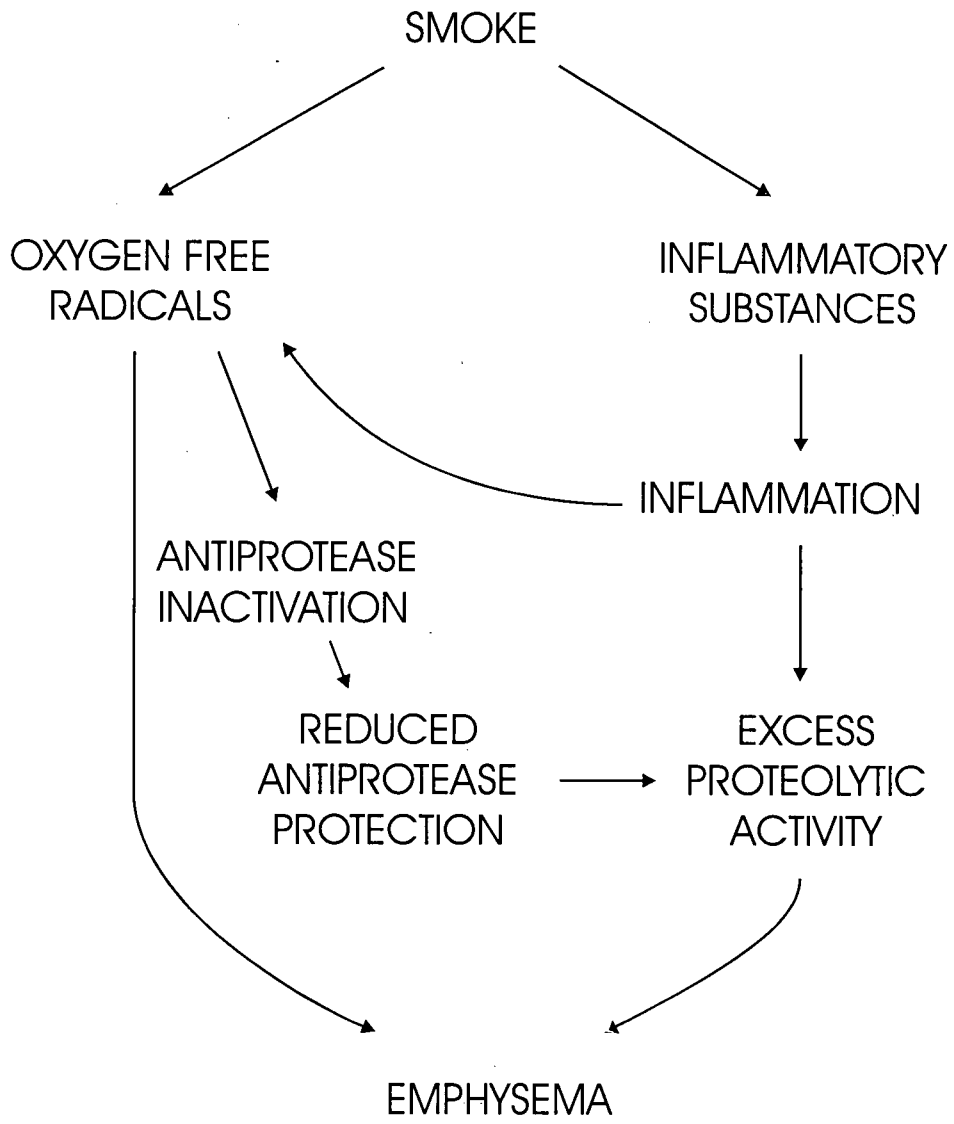

Figure 1.1- Schematic summary of protease-antiprotease interaction in smoke-induced emphysema.
Cigarette smoke is demonstrated to contain oxygen free radicals and other substances which induce inflammation in the lung. This culminates in complex interactions (depicted by arrows, pointing in direction of cause to putative effect), ultimately leading to proteolytic imbalance and emphysema.

A potential role for proteases in emphysema: α_1-antitrypsin deficiency

In 1965, Laurel and Eriksson identified an association between familial deficiency in α_1-antitrypsin (α1AT) and increased susceptibility to development of emphysema at an early age (reviewed in Eriksson, 1989). α1AT is synthesized primarily by hepatocytes in the liver, and is the major plasma serine proteinase inhibitor. Approximately 75 alleles at the α1AT locus have been identified (reviewed in Crystal, 1989). The "normal" allele is the M allele (M1-M4). Homozygotes for the M allele have serum α1AT concentrations between ~20 to 50 μM. Twelve 'at-risk' alleles have been identified which result in reduction or complete deficiency of serum α1AT. A serum level of less than ~11 μM predisposes an individual to emphysema. The most common at-risk alleles are the Z (allelic frequency, 0.01-0.02), and S (allelic frequency, 0.02-0.04) alleles*. ZZ homozygotes have α1AT serum levels between ~3 and 8 μM and are at risk for disease. SZ heterozygotes (~8 to 22 μM serum α1AT), but not SS homozygotes (~17 to 33 μM) are at mild risk for disease. Interestingly, not every α1AT-deficient individual appears to be at equal risk for development of pulmonary disease: Approximately 52% of non-smoking ZZ homozygotes develop COPD and 84% of ex-smoking α1AT-deficient individuals show signs of COPD (reviewed in Eriksson, 1996). The mechanisms which allow 48% of non-smoking homozygotes and 16% of ex-smoking homozygotes to escape the disease are not clear, and may reflect the factors which allow for differences in susceptibility to smoking-associated (acquired) emphysema. A relationship between the severity of emphysema in α1AT-deficient individuals and the quantity of NE carried in their neutrophils (Hubbard et al. 1990) has been suggested, but is unlikely to be the only factor involved.

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* Allelic frequencies for Z and S alleles are for Caucasians of European descent; these alleles are rare in other populations.

Kinetic studies have demonstrated a high rate of association between α1AT and NE. Importantly, NE is also the principal substrate for α1AT, indicating that the primary physiological role of α1AT may be to inhibit NE (Crystal, 1989) These observations imply that NE is involved in the pathogenesis of α1AT deficiency induced emphysema. Numerous studies have attempted to extend this observation to the smoke-induced form of emphysema, suggesting that NE may be important to the development of cigarette-smoke induced emphysema. While many findings have supported the protease-antiprotease hypothesis in general (see below), no definitive evidence for either a dominant or a necessary role of NE (or of any other specific protease) in the development of acquired, smoke-induced emphysema has yet been obtained.

Pulmonary inflammatory cells in emphysema

In determining the potential role of proteolytic activity in smoke-induced emphysema, studies describing the source and nature of proteases have provided much information. The dominant source of destructive extracellular proteases in the lung are inflammatory cells. In both the normal and emphysematous lung, the pulmonary interstitial and alveolar macrophages and neutrophils are the most abundant inflammatory cells. Alveolar macrophages are phagocytic cells that reside in the alveolar space; they are the most abundant immune cells in this compartment, comprising greater than 90% of cells derived from bronchoalveolar lavage (BAL) of the normal lung (Reynolds, 1987). Alveolar macrophages are the initial and predominant line of defense in the lung against inhaled micro-organisms and debris and also engulf expired neutrophils which have extravasated into the pulmonary airspace. A substantial assortment of anti-microbial substances, including proteases, oxidants, inflammatory mediators, and chemoattractants, are actively produced in the alveolar macrophage (Table 1.1). Although they are normally sufficient on their own to contend with inspired microbes, when overwhelmed, alveolar macrophages are able to induce a rapid efflux of neutrophils from the pulmonary vasculature into the lung.

Table 1.1 Proteases in the lung

source	proteases			
neutrophils	neutrophil elastase	(s)	[+++]	(2-5 μg/10^6 cells)
	proteinase-3	(s)	[+++]	
	cathepsin G	(s)	[++]	(1-2 μg/ 10^6)
	lysozyme			
	type-I collagenase	(m)		
	92 kDa gelatinase	(m)		
	cathepsin B	(c)	[+/-]	(1ng/10^6)
	cathepsin C (dipeptidyl peptidase I)			
	cathepsin D	(a)		
	heparinase			
	plasminogen activator	(s)		
alveolar macrophages	macrophage elastase	(m)		
	collagenase	(m)		
	stromelysin	(m)	[++]	
	lysozyme			
	92 kDa gelatinase	(m)	[+/-]	
	cathepsin B		[+]	(0.2μg/10^6)
	cathepsin D			
	cathepsin H	(c)		
	cathepsin L	(c)	[+/-]	(0.03 ug/10^6)
	cathepsin S	(c)		
	matrilysin	(m)	[+/-]	
	plasminogen activator			

Abbreviations: Symbols in brackets indicate the relative abundance of these proteases in their respective cells, where reported. [+++] indicates the greatest abundance, with over ~2μg per 10^6 cells, [++] indicates ~1 to 2 μg per 10^6 cells; [+], ~0.1 to 1 μg per 10^6 cells, and [+/-] indicating lower amounts. In parentheses: c, cysteine protease; m, metalloprotease; s, serine protease. Also indicated is the mass (in micrograms) per 10^6 cells, where known.

Neutrophils are normally confined to the intra-vascular compartment of the lung. Approximately 2.5×10^9 neutrophils are produced per hour in the bone marrow, have a half-life of approximately 6 to 7 hours in peripheral blood, and constitute 60-70% of all circulating leukocytes (Sibille and Marchandise, 1993). Although neutrophils constitute only a small percentage of cells obtained in BAL (2 to 4%), there are approximately 10^8 neutrophils in the pulmonary vasculature at a given time, representing the largest pool of neutrophils in the body (MacNee and Selby, 1993). The extreme proximity between the alveolar epithelium and lumen of the pulmonary capillaries permits a rapid extravasation of neutrophils into the intra-alveolar space when needed. Neutrophils carry an extensive array of proteolytic enzymes (Table 1.1) and oxidants in their azurophilic (primary) and specific (secondary) lysosomal granules. These granules are used in the formation of phagolysosomes and are also capable of degranulating, releasing their granule constituents into the micro-environment. While alveolar macrophage proteases are constitutively produced in a regulated fashion, mature neutrophils carry their entire arsenal of destructive enzymes within their granules upon exiting the marrow. The contents of these granules can be released into the local micro-environment through active exocytosis (Wright, 1988; Fallon and Gallin, 1986), frustrated phagocytosis, and possibly through leakage from spent, apoptotic neutrophils which have not been removed by alveolar macrophages.

Smoking increases the total inflammatory cell content of the lung (Wallace and Lamb, 1992), thereby increasing the proteolytic load in the lung. The alveolar macrophage population is chronically increased in smokers as determined from BAL, and remains the most abundant cell in the intra-alveolar space subsequent to cigarette smoking. Respiratory bronchiolitis can be observed in the lungs of young smokers (without emphysema), where alveolar macrophages are concentrated in the vicinity of the respiratory bronchiole (Niewoehner et al. 1974), temptingly suggesting a causative effect of these cells in mediating the destruction of the acinus in this area in centrilobular emphysema. The increased alveolar macrophage population in chronic smokers is believed to stem from recruitment of peripheral blood

monocytes, possibly from differentiation of interstitial macrophages, or from proliferation of existing alveolar macrophages (Thomas et al. 1976; Golde et al. 1974).

Neutrophils are also increased in the intra-alveolar space of smokers (Morrison et al. 1998; Markos et al. 1993); however, the relative proportion of neutrophils to alveolar macrophages derived in BAL does not change substantially. The intra-alveolar neutrophil increase in cigarette smoking is accompanied by an increase of the more substantial population of neutrophils within the intra-vascular space (MacNee et al. 1989). The effect of smoke-induced neutrophil accumulation has been experimentally verified in animals which also indicate that a greater percentage of sequestered neutrophils induced by smoking consist of newly synthesized cells (Terashima et al. 1999), implying either a preferential sequestering of new cells or stimulation of neutrophil production in the marrow as a consequence of smoking. Interestingly, nicotine has been shown to have chemotactic activity (Totti et al. 1984) and to delay neutrophil apoptosis *in vitro* (Aoshiba et al. 1996); the relative contribution of these effects to the total lung neutrophil population however, has not been determined. Sequestering of neutrophils in the lung secondary to smoking is influenced in part by delayed passage of pulmonary neutrophils due to microvascular compression secondary to increased airways resistance during smoking (Bosken et al. 1991; Markos et al. 1993). Smoke-induced decrease in deformability of the neutrophil may also slow the passage of neutrophils through the lung (Drost et al. 1992). Although no reports are available describing the importance of neutrophil-endothelial cell adhesion secondary to smoking and emphysema, adhesion to endothelial cells is likely to contribute to marginalization of the neutrophil in the lung. Neutrophil adhesion due to stimulation by chemotactic factors has been well studied in other systems. In the lung, neutrophil adhesion may be stimulated by release of chemotactic factors from activated alveolar macrophages secondary to cigarette smoking (Hunninghake and Crystal, 1983).

Increased neutrophil and macrophage populations associated with acute and chronic smoking suggests a potential role for one or both of these cell types in the development of emphysema. Endotoxin mediated sequestration of leukocytes in experimental animals has been demonstrated to induce mild em-

physema (Wittels et al. 1974; Guenter et al. 1981), as has FMLP-induced neutrophilia (Cavarra et al. 1996) and intratracheal instillation of lipopolysaccharide (LPS) (Stolk et al. 1992). These observations demonstrate the potential of neutrophil sequestration to produce emphysema. Studies in humans addressing a possible correlation between chronic increase in neutrophil or macrophage populations and presence and severity of emphysema have provided varying data: In a cross-sectional study of lung tissue no significant relationship was observed between the presence of emphysema and the percentage or numbers of inflammatory (PMN or monocytic) cells per mm^2 total tissue area (Wright, 1988). A similar study reported a direct correlation between numbers of alveolar macrophages and T-lymphocytes (per mm^3 lung parenchyma) with presence of emphysema, whereas the presence of neutrophils correlated negatively with the extent of airspace enlargement (Finkelstein et al. 1995). These studies suggest that neutrophils do not have a role in the development of emphysema, and seem to be inconsistent with the aforementioned smoking-induced inflammatory cell increase. Another study examining neutrophils per mm (length) alveolar wall (as opposed to mm^2) has reported a slight increase in neutrophils in smokers with emphysema (Ludwig et al. 1985). A significant issue with such cross-sectional studies is that they do not account for possible transient increases in pulmonary neutrophils in individuals with emphysema which arise during cigarette smoking or shortly thereafter. Acute episodes of inflammation following smoking also would not be observed. Cross-sectional studies only preclude the possibility that neutrophils or alveolar macrophages have a role in inducing emphysema through a chronic, steady-state increase in cell numbers. A final consideration is that an increase (or decrease) in inflammatory cell population in emphysema could be considered a consequence rather than cause of development of emphysema. Thus, determining whether such an increase precedes disease development at an early stage would have more significant implications in indicating the contribution of these cells to the development of the disease in humans.

Although the neutrophil is capable of inducing experimental emphysema (above), whether the neutrophil or the alveolar macrophage is more important in the development of emphysema, and indeed, whether either of the two are absolutely essential to the development of emphysema cannot be inferred from the existing data. A large number of studies have focused instead on individual proteases within these two cell types.

Proteases and emphysema

According to the protease-antiprotease hypothesis, proteolytic destruction of the extracellular matrix (ECM) is an obligatory step in the pathogenesis of emphysema. Although many proteases are found in the lung (Fig 1.2), the discussion here is restricted primarily to inflammatory cell proteases with elastolytic activity. Cells of the lung parenchyma are also known to produce proteases, but the majority of these cells are thought to be involved in normal connective tissue turn-over. Inflammatory cells are the primary source of extracellular proteases in the lung, consisting predominantly of alveolar macrophages in the intra-alveolar space and of neutrophils in the intra-vascular space, both of which become more abundant subsequent to cigarette smoking, as discussed above. There is substantial overlap between the protease content of neutrophils and alveolar macrophages (Table 1.1). This not surprising, since they are derived from a common myeloid progenitor; however, there are also a number of proteases specific to each of these two cell types. The armament of proteases produced and carried by the alveolar macrophage and neutrophils are capable of degrading most components of the lung ECM (Table 1.2). Not all macrophage and neutrophil proteases have necessarily been identified, nor have their abundance, kinetics, and substrate specificity been accurately compared using standardized assays; however, the most abundant proteases and therefore those with the greatest potential implications in protease-antiprotease imbalance leading to disease have likely been identified. A number of observations have lead to the

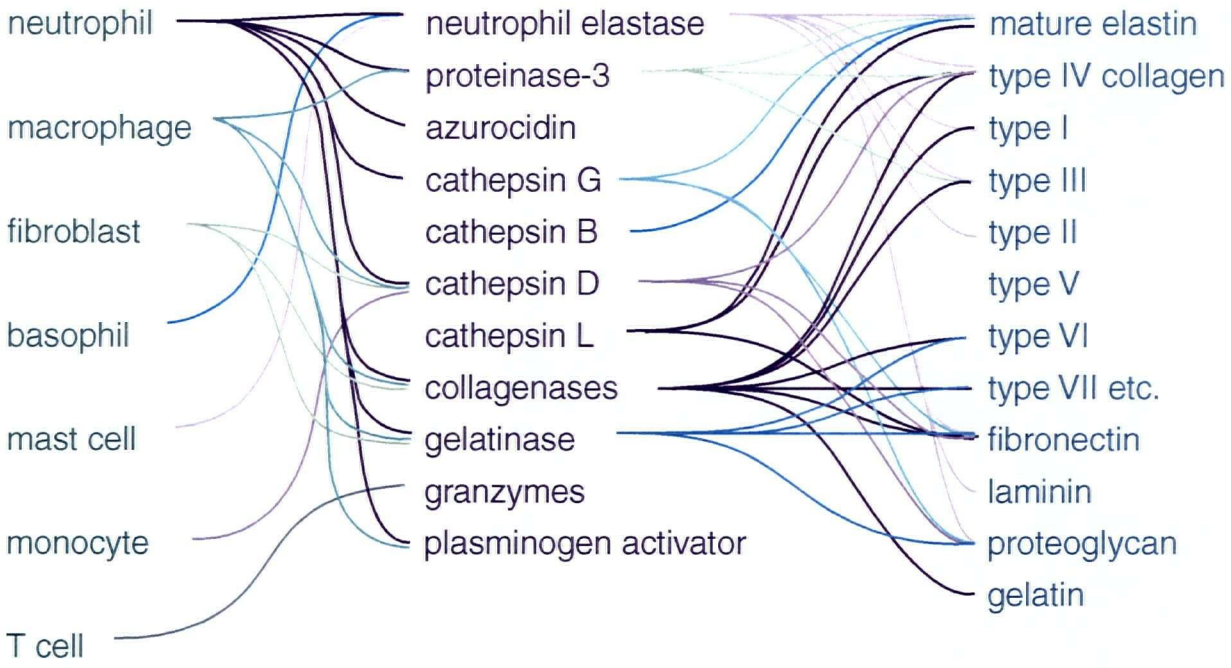

Figure 1.2- Simplified schematic depicting some of the complex interactions and redundancies between cells and their proteases, and between proteases and their respective substrates in the lung.
Colored lines show some of the identified relationships between the indicated cells and proteins; with proteases (center) from cells (left), cleaving their known substrates (right). Since these interactions are summarized from different sources (see text), relative/ quantitative relationships are not shown, nor have all the interactions between the listed substances necessarily been studied. Potential involvement of antiproteases and other non-proteolytic factors are also not shown. A key concern in the relationship between these proteases and emphysema would therefore be to understand which factors are likely to be the *most important* factors, rather than trying to identify *all* the possible factors in the disease.

Table 1.2 Neutrophil and macrophage protease substrates.

	elastin	collagen I	collagen II	collagen III	collagen IV	fibronectin	gelatin	laminin	proteoglycans
NE	+	+	+	+	+	+		+	+
PR3	+	+		+					
CatG		+		+	+	+		+	+
ME	+								
collagenase		+		+	+	+			
gelatinase	+				+	+	+	+	
matrilysin	+								
stromelysin	+	+							

Abbreviations: (+), reported substrates are shown for the indicated proteases where studied. Not all substrates were examined in the studies from which this table was constructed; importantly, the conditions (buffer, pH, etc.) in which they were examined differ from study to study and can presumably alter the substrate specificity. This table should therefore be interpreted as being reflective of primary substrate specificity for each enzyme.

generally accepted view that destruction of elastin in emphysema is an essential and/or limiting factor in the development of emphysematous lesions:

1. destruction of elastic tissue is observed in emphysematous lesions (discussed above),
2. decreased elasticity is observed in the severely emphysematous lung,
3. NE is important in the development of α1AT-deficiency induced emphysema (discussed above),
4. elastin-derived peptide levels are elevated in plasma (Akers et al. 1992) and urine (Schriver et al. 1992) of individuals with COPD
5. elastolytic but not non-elastolytic proteases are capable of inducing experimental emphysema via intratracheal instillation* (Kao et al. 1988; Snider et al. 1974, and below). Non-elastolytic proteases such as pancreatic trypsin or bacterial collagenase cause acute lung injury, but the lungs heal completely after the injury and emphysema is not induced.

Direct comparison of elastolytic or of other proteolytic activities is hindered by the different conditions (pH, presence of other potentiating co-factors or enzymes, substrate specificity, etc.) that are required for optimal activity for each protease; however a rough order of elastolytic potential has been extrapolated based on comparisons extracted from individual reports (Chapman et al. 1994):

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* The requirement of elastolytic activity in inducing emphysema is a general observation in experimentally induced emphysema, but recently exceptions have been found; e.g. intratracheal instillation of hyaluronidase and subsequent exposure to an otherwise non-toxic concentration of oxygen (60%) for 4 days leads to airspace enlargement with decrease in total lung elastin (Cantor et al. 1993). Cadmium chloride has also been used to produce emphysema (Snider, 1992b).

cathepsin L (pH5.5) > cathepsin S (pH5.5) >> NE (pH 7.4) > cathepsin S (pH7.5) ≈ 72 kDa gelatinase > matrilysin ≈ proteinase 3 (pH 7.4) > macrophage elastase ≈ cathepsin G > 92 kDa gelatinase

Acid proteases such as cathepsins L and S, though extremely active in the acidic environment of the phagolysosome, have greatly reduced proteolytic activity in the neutral pH of the lung. Of the neutral proteases, it can be seen that NE is the most elastolytic. NE also has a particularly broad substrate specificity; in addition to elastin, it has been demonstrated to hydrolyze most ECM components, including types I, II, III, IV, VIII, IX, X, and XI collagen (Kittelberger, 1992; Gadher and et al., 1988), and glycoproteins such as fibronectin, proteoglycans, and laminin. More than 45 proteins have been identified which are susceptible to cleavage by NE; in consideration of its abundance in the neutrophil (3-5 μg/ 10^6 cells) (Jochum et al. 1994) and the abundance of the neutrophil in the lung*, NE is of apparent significance in the context of the protease-antiprotease hypothesis of emphysema, even in the absence of further incriminating evidence. The role of NE in α1AT-related disease further substantiates its putative role in protease-mediated lung injury.

In consideration of the potential of NE as a contributor to proteolytic activity in the lung, many studies have attempted to provide evidence of a specific role of NE in the development of emphysema. Attempts to correlate the presence of NE with emphysema have produced varying results: Direct immunohistochemical localization of elastase demonstrates the presence of NE bound to patches of elastin in centri-

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* Under normal conditions, with ~6 x 10^7 neutrophils in the lung, and considering that there are ~3 to 5 pg of NE/ cell, a simple calculation reveals that ~200 to 300 μg of total NE are in the lung at a given time. This amount would be increased during neutrophil retention in smoking and inflammation.

lobular emphysematous lesions and a rough correlation (r=0.81) between presence of elastase and mean linear intercept has been reported (Damiano et al. 1986), confirming the possibility of a direct role of NE in degrading the elastic fibres in emphysematous lesions. This study cannot, however, suggest the relative contribution of NE to degradation of elastin in these lesions in comparison to other destructive agents. There is a direct correlation between BAL elastase burden (indicated by immunogenic NE/α1AT complex) and severity of emphysema as determined by computed tomography (Fujita et al. 1990), supporting an important role of NE in development of emphysema. Increased NE is observed as a consequence of smoking in general: A study of immunogenic NE derived from BAL indicates that smoking induces an acute, transient increase in the levels of NE in the upper lobes of the lung (Abboud et al. 1987). Elevated BAL-derived elastin-derived peptide levels are observed in smokers versus non-smokers and appear to correlate with immunogenic NE (Betsuyaku et al. 1996), consistent with the interpretation that increased NE in smoking effectively degrades elastin in the presence of the usual excess α1AT in BAL. Although the bulk of NE obtained from BAL in these experiments is bound to α1AT and therefore inactive, the possibility remains that NE acts primarily in the microenvironment of the neutrophil is not excluded by this observation. A hypothetical antiprotease-free zone in the vicinity of the neutrophil during degranulation could be created by oxidative processes (see below) and by other proteases that are able to inactivate α1AT. Additionally, free neutrophil elastase has been detected in the epithelial lining fluid (ELF) of some cigarette smokers (Stockley and Burnett, 1979), but these are exceptions. In mice, serine protease elastolytic inhibitory capacity* in different strains has been demonstrated to correlate inversely with experimental emphysema induced by FMLP-induced neutrophilia (Cavarra et al. 1996), further supporting the role of neutrophils and NE in producing emphysema through proteolysis.

By virtue of its broad substrate specificity, in addition to its ability to directly damage lung parenchyma, NE may indirectly affect numerous physiological functions. For instance, NE may potentially

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* Elastase inhibitory activity was determined by titration with mouse neutrophil elastase.

modulate various inflammatory responses through cleavage of coagulation factors (fibrinogen, factors V, VII, XII, and XIII), plasminogen, IgG, IgM, complement factors C3 and C5, *E.coli* cell walls, and gp120 HIV coat protein (reviewed in Gillis et al. 1997). It also induces IL-8 gene expression in epithelial cells (Nakamura et al. 1992). The NE/α1AT complex is also chemotactic (Banda et al. 1988). The overall significance of these typically isolated observations to the pathogenesis of emphysema however, is unclear.

There is evidence in an experimental model that elastolytic activity in alveolar macrophages increases upon exposure to cigarette smoke (Hinman et al. 1980; Ofulue et al. 1998). Although elastolytic activity in the macrophage has long been recognized, there has been controversy until recently as to whether this elastolytic activity was from macrophage-bound/ internalized NE, or whether it was a novel elastase. Macrophage elastase (MoE), a protease distinct from NE, has since been shown to contribute to the bulk of alveolar macrophage elastolytic activity (Senior et al. 1989) and is upregulated following cigarette smoke in vivo (Hautamaki et al. 1997). Antithetically, another group has provided some evidence that elastolytic activity in macrophages is due primarily to a cysteine protease, perhaps cathepsin S (Chapman et al. 1994). Although its activity is reduced under neutral conditions, significant elastolytic potential of cathepsin S remains at pH 7.5. Recently, the murine MoE gene was genetically inactivated via gene-targeting (Hautamaki et al. 1997). Exposure of control mice to cigarette smoke for six months resulted in mild airspace enlargement, as indicated by measurements of mean linear intercept (Lm). Homozygous deficient (-/-) MoE mice exhibited a diminished increase in pulmonary macrophage population and reduced airspace enlargement consequent to smoking, relative to controls. These results indicate a role for MoE in experimental emphysema in mice, and suggest a contribution of MoE to the pathogenesis of emphysema in humans.

Previously, the possibility of an important role of a macrophage protease in emphysema had seemed unlikely as production of macrophage proteases, unlike those of the neutrophil, are regulated and can be produced in the mature cell. Furthermore, ECM destruction by macrophages requires contact with

the surface of the substrate (Senior et al. 1989). As alveolar macrophages normally only come in contact with the alveolar epithelium and not the ECM, it is not immediately apparent how macrophage proteases contribute to elastin damage. Alveolar macrophages have also been demonstrated to secrete antiproteases against metalloproteases and cysteine proteases which would presumably inactivate MoE or cathepsin S; the relevance of this is unknown. A more fundamental concern is whether MoE contributes directly to smoke-induced airspace enlargement, or whether it acts indirectly, for instance, by accelerating tissue damage, by inactivating α1AT or other antiproteases, by priming the ECM for further damage by other proteases, through recruitment of other inflammatory cells, etc. To address these possibilities, the ability of MoE to induce emphysema in experimental animals needs to be demonstrated. Furthermore, to assess the relative contribution of MoE to emphysema, the effect of NE and other suspected proteases should be similarly demonstrated through gene targeting. A direct comparison of the relative contribution of the various suspected proteases in the development of experimental emphysema will provide a good indication of which protease(s) are of paramount importance to development of the disease.

Although NE and MoE have been especially well studied because of their quantity, function, or other observations which have supported their role in emphysema, other proteases found in both the neutrophil and macrophage also have the potential of causing tissue damage, though they have been less extensively studied: Proteinase-3 (PR3) is elastolytic, structurally similar to NE, and has been shown to induce experimental emphysema through intratracheal instillation (Chapter 4 and Kao et al. 1988). Cathepsins S and L are also elastolytic. Non-elastolytic proteases may also have potential roles in destruction of lung parenchyma, either directly, or by potentiating the effects of another protease. Cathepsin G for instance, is present in the neutrophil in amounts only slightly less than NE, and though it has little elastolytic activity on its own, it cooperatively enhances the development of experimental emphysema when administered intratracheally with elastase (Lucey et al. 1985; Sloan et al. 1981). Collagenases are found in both the neutrophil and macrophage, and are capable of hydrolyzing helical collagen fibrils in

the lung. Recent evidence suggests that interstitial collagenase (matrix metalloproteinase-1, MMP-1) is a significant requirement for the induction of experimental emphysema (below). Expression of gelatinase B and interstitial collagenase (but not MoE) in BAL-derived alveolar macrophages is elevated in smokers and ex-smokers with emphysema versus a matched population of smokers without emphysema (Finlay et al. 1997a; Finlay et al. 1997b). Administration of trypsin subsequent to elastase impairs elastin synthesis in the lungs of hamsters with onset of experimental emphysema (Osman et al. 1985). Intravenous injection of trypsin into rats has been shown to induce experimental emphysema* (Reichart et al. 1992). Although the ability of NE to induce experimental emphysema through proteolysis is not unique, the intrinsic properties of NE and numerous indirect evidences supporting its involvement in emphysema have distinguished it from most other proteases in the lung in this disease.

Antiproteases

Emphysema associated with α1AT deficiency has remained the most convincing evidence of the protease-antiprotease hypothesis; however, most studies examining this hypothesis have focused on excessive proteolytic activity rather than reduced antiproteolytic protection. This focus on proteases is due in part to the observation that most antiproteases are present in much greater than molar equivalence to their corresponding proteases. It is also more convenient experimentally, to increase the protease-antiprotease ratio by increasing levels of proteases than by depleting antiproteases. Most antiproteases furthermore exhibit multifarious inhibitory capacities for a number of proteases and would thus complicate any interpretations resulting from experiments in which an antiprotease is the variable. In any event, most of the arguments for and against the protease-antiprotease theory as described in the previous sec-

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* An obvious sequestration of granulocytes in the pulmonary microvasculature occurs in this model, and hence it is not clear that trypsin directly causes the airspace enlargement

tion regarding proteases in emphysema also indicate the potential of antiproteolytic involvement in emphysema. A few features of particular interest should be mentioned. Several antiproteases are present in the lung at significant levels and are generally in excess of lung proteases in both non-smokers and in smokers. Pulmonary surfactant itself is believed to exhibit antiproteolytic properties (Otto-Verberne et al. 1991). Possibilities as to how proteases may evade antiprotease inactivation during smoking in some individuals include (i) proteolytic cleavage or oxidative inactivation of antiproteases in the local microenvironment of the inflammatory cell, (ii) exclusion of antiproteases by direct surface-mediated degradation by neutrophils and macrophages, and (iii) proteolytic function in areas which are sterically inaccessible to the antiprotease of concern. No antiprotease has appeared to be singularly important in the prevention of acquired emphysema; however, α1AT and secretory leukoprotease inhibitor appear to be particularly abundant in the lung.

α1AT is the predominant extracellular serine proteinase inhibitor in serum and one of the most abundant antiproteases in epithelial lining fluid (ELF). The rate of association between α1AT and NE is 3 orders of magnitude greater than the rate of association between α1AT and trypsin and 2 orders of magnitude greater than the rate of association between α1AT and cathepsin G (summarized in Table 1.3). The high rate of association emphasizes the specificity of α1AT for NE and suggests that specific mechanisms may have evolved to counteract the particularly destructive effects of NE. α1AT is responsible for greater than 90% of the anti-neutrophil elastase function in the lower respiratory tract (Hubbard and Crystal, 1992), and NE derived from BAL is completely bound and inactivated by α1AT under normal circumstances and in smoking (see above); however, this does not exclude the possibility that NE is transiently active in the pericellular environment of the neutrophil.

Table 1.3 Rate of association of proteases with α_1-antitrypsin.

Enzyme	Association rate ($M^{-1}sec^{-1}$) with α_1-antitrypsin
neutrophil elastase	10^7
trypsin	10^4
cathepsin G	10^5

Along with α1AT, secretory leukoprotease inhibitor (SLPI; bronchial mucous inhibitor, antileukoprotease, see Table 1.4) is also present at significant levels in the upper respiratory tract. Although SLPI has a similar rate of association with NE as α1AT, it is present at lower concentrations in the lower respiratory tract (Hubbard and Crystal, 1992). Both SLPI and elafin (see below) are capable of inhibiting elastase activity of neutrophils even when neutrophils are bound to substrate, unlike α1AT (Rice and Weiss, 1990), suggesting an important role of these antiproteases in preventing neutrophil protease mediated lung injury. Intratracheal SLPI supplementation in experimental animals ameliorates LPS-induced pulmonary emphysema (Rudolphus et al. 1993), suggesting that a serine protease is involved in neutrophil-mediated lung injury.

Alpha-2-macroglobulin (α2MG) is a third antiprotease found in the lung; it is a non-class specific protease inhibitor and has an association rate with NE that is similar to that of α1AT (Hubbard and Crystal, 1992). Although α2MG is normally present in the epithelial lining fluid (ELF) of the lung at levels ~200 to 600-fold less than that of α1AT, it is produced by alveolar macrophages, indicating that its concentration in the vicinity of the alveolar macrophage would be substantially greater than suggested

Table 1.4- Antiproteases in the lung.

antiprotease	substrate	source
α_1-antitrypsin	serine: neutrophil elastase, cathepsin G	hepatocytes (primary source)
α_1-antichymotrypsin	serine: cathepsin G	hepatocytes
α_2-macroglobulin	all four classes of proteases	hepatocytes, alveolar macrophage, monocytes, fibroblasts
secretory leukoprotease inhibitor (SLPI)	serine: neutrophil elastase, cathepsin G	mucous cells
elafin (low molecular weight elastase-specific inhibitor)	neutrophil elastase	bronchial epithelial cells
tissue-inhibitor of metalloproteinase (TIMP)	metallo: macrophage elastase, collagenase, stromelysin	alveolar macrophage, fibroblasts
cystatin C	cysteine proteinases (cathepsins B, H, L, S)	alveolar macrophage

Note: Substrates listed are potentially destructive proteases found in the lung (see Table 1.2).

by its measured inhibitory capacity in BAL. α2MG may thus have a significant role in preventing local damage by alveolar macrophage proteases. α2MG levels can also increase with increases in alveolar epithelial permeability.

Elafin (also elastase-specific inhibitor) is a recently identified reversible serine proteinase inhibitor with specificity for NE (Schroder et al. 1990). Elafin was isolated originally from the skin, but has been found in bronchial secretions, and is expressed by Clara cells and type II alveolar cells (Sallenave et al. 1993). Tumor necrosis factor is a strong inducer of elafin in a type II alveolar cell line, suggesting that induction of expression of elafin may be important in preventing tissue damage by NE during inflammation.

A number of studies have attempted to describe mechanisms of antiprotease inactivation. For instance, α1AT, tissue inhibitor of metalloproteinase (TIMP), and cystatin C can be cleaved by proteases, α1AT is inactivated by oxidant species, etc. It remains unclear however, how much such mechanisms can affect the overall antiprotease screen considering the large molar excess of most antiproteases. Cleavage of antiproteases may momentarily potentiate local, pericellular activity of neutrophil proteases upon degranulation or after phagolysosome formation and this may be an important mechanism in permitting proteolysis in a localized area. Aberrant inactivation of antiproteases is a possible route leading to ECM injury, but this has been difficult to prove experimentally with respect to emphysema.

Oxidants and emphysema

Oxidants in the lung, particularly reactive oxygen species (ROS) and nitrogen oxides (NO_X), are potential direct mediators of lung injury and are also capable of affecting antiprotease activity. Oxidants thus have potential roles in the pathogenesis of emphysema. Demonstration of possible involvement of oxidants in emphysema are complicated by the short life span of highly reactive oxidant species, the non-

specific nature of oxidant interactions, and the numerous physiological responses which occur with or as a result of oxidant activity.

There are two abundant sources of oxidants in the lung: exogenous oxidants are found in ambient air and in cigarette smoke while endogenous oxidants are derived from normal metabolism and from the neutrophil and alveolar macrophage. Possible roles of oxidants in cigarette smoke induced lung injury are described here; overviews of oxidant chemistry and biochemistry can be found in standard textbooks. Cigarette smoke consists of a gas and tar phase, both of which contain oxidant species. In modern cigarettes, greater than 99.9% of the tar phase is removed. The gas phase of cigarette smoke contains up to 10^{17} free radicals per puff (Pryor, 1987) and ~1000 ppm NO_X (Ames et al. 1993). Free radicals in the gas phase are known to have fleeting life-spans of approximately 1 second; however, ROS are detected maximally in the gas phase at 1 to 2 minutes. This discrepancy is proposed to arise from the continuous production of ROS by nitric oxide (NO·) in the gas phase (Church and Pryor, 1992); carbonyl sulfide has also been proposed to play a role in generation of oxidative species in the gas phase (Kodama et al. 1997). Neutrophils and macrophages are the primary source of endogenous oxidant activity in the lung; both release the superoxide anion (O_2^{-}·) and nitric oxide (NO·). Neutrophils also carry myeloperoxidase, which contributes to the generation of hypohalous acids and chloramine. Oxidant species of varying reactivities can be interconverted spontaneously or in the presence of catalysts, enzymes, and reagents: ($O_2 + e^- \rightarrow O_2^{-}\cdot + e^- \rightarrow H_2O_2 + e^- \rightarrow \cdot OH + e^- \rightarrow H_2O$).

Dedicated and "inadvertent" antioxidants are found both intracellularly and extracellularly in the lung. Glutathione (GSH) is the predominant small antioxidant molecule in the ELF of the lung, with concentrations exceeding that in serum by 100-fold. Extracellular catalase, as well as low levels of antioxidant molecules such as ascorbate, vitamin E, A, and C, and ferritin are also found in the lung (Heffner and Repine, 1992). Serum proteins such as albumin, ceruloplasmin, and transferrin are found in the ELF at about a tenth of the concentration in serum; however, albumin still accounts for approximately half the

protein content in ELF, and acts as a sacrificial antioxidant by reacting with highly reactive oxidant species before they damage more important molecules or cell membranes. Intracellular antioxidants such as superoxide dismutase (SOD), catalase, glutathione reductase and glutathione peroxidase are less likely to play a role in the development of emphysema. Erythrocytes may be of some importance as they are permeable to H_2O_2 and O_2-, are abundant, and possess an effective antioxidant system (Toth et al. 1984; Agar et al. 1986).

Oxidants could potentially damage lung parenchyma through several routes:

1. impairment of antiprotease defense,
2. direct cytoxicity or damage of lung ECM,
3. recruitment of inflammatory cells or activation of existing cells,
4. impairment of tissue repair.

There is some evidence supporting each of these possibilities; however, the overall contribution of oxidant-mediated damage to the development of smoke-induced emphysema is not well understood. The short life-span of most ROS make their direct detection in vivo difficult or impossible and their presence is generally inferred indirectly through the presence of by-products of oxidant activity (i.e. oxidized methionine, lipid peroxidation, soluble extracellular molecules).

There is evidence that antioxidant levels are generally decreased subsequent to cigarette smoking. For instance, GSH levels are transiently decreased for several hours following smoking, suggesting oxidant stress resulting from cigarette smoke exposure (Li et al. 1996). Vitamin E levels are also reduced due to conversion to an oxidized product (Pacht et al. 1986). Oxidative inactivation of α1AT leading to decreased elastase inhibitory capacity in smokers has been demonstrated (Carp et al. 1982). Cigarette smoke contributes directly to the oxidative inactivation of α1AT (Janoff and Carp, 1977). NO_2 or NO_2-derived ROS in smoke may be involved in this process (Hood et al. 1993). Alveolar macrophages of cigarette smokers have also been shown to oxidatively inactivate α1AT (Hubbard et al. 1987;

Pryor, 1987). The overall significance of oxidative stress in emphysema is unknown. A complicating factor is that direct evidence of oxidative damage *in vivo* leading to emphysema has been difficult to ascertain.

Direct damage of lung tissue by oxidants has been demonstrated *in vitro*. Exposure of rat tracheal explants to cigarette smoke alone produces lipid peroxidation products in the absence of inflammatory cells (Churg and Cherukupalli, 1993). Hydrogen peroxide from activated macrophages and granulocytes is cytotoxic *in vitro* (Nathan et al. 1979; Weiss et al. 1981). Indirect damage mediated by oxidant activity is also possible. For instance, recruitment of inflammatory cells by oxidants has been demonstrated *in vivo*. Exposure of rats or hamsters for a six week period to 30 ppm NO_2, an oxidant and free radical generator in cigarette smoke, is characterized by an initial, acute 200-fold increase in neutrophils, decreasing to a 20-fold overall increase by 7-days, and resulting in emphysema (Freeman et al. 1968; Glasgow et al. 1987; Lam et al. 1983). This observation indicates that the NO_X component of cigarette smoke can directly lead to emphysema, possibly due to a chronic inflammatory reaction induced by these substances.

A specific model of emphysema associated with oxidation can be observed in the silica-induced emphysema model. Although emphysema arising from mineral dust exposure accounts for only a small percentage of patients with emphysema, the etiological agent is simpler than cigarette smoke and provides some evidence of how oxidative damage may contribute to the disease. A model of silica (quartz)-induced emphysema has been produced in rats (Wright et al. 1988) with evidence of elastin and collagen proteolysis accompanied by a 170-fold increase in BAL neutrophil population and a 2-fold increase in the macrophage population (Li et al. 1996). Quartz has been demonstrated to generate H_2O_2 *in vitro*, and has the capacity to oxidatively inactivate α1AT (Zay et al. 1995). Together, these observations suggest that oxidants from silica alone are able to induce an inflammatory influx, leading to emphysema. Interestingly, silica exposure is also associated with fibrosis (the same is true with exposure to $CdCl_2$ or O_3), suggesting possible similarities between the mechanisms leading to fibrosis and to emphysema.

Although the extent to which oxidants contribute to the development of emphysema and their relation to proteolytic activity in the lung is still poorly understood, oxidants alone appear to be capable of inducing lung-injury in animal models. Oxidant-mediated lung injury may be an important instigating factor in emphysema with direct cytotoxic effects and perhaps indirectly leading to further damage by potentiating proteolytic activity. There is little evidence which suggests that oxidants alone directly lead to the bulk of damage found in emphysematous lesions in humans.

A supplementary theory: 'inflammatory-repair'

The 'inflammatory-repair' hypothesis of emphysema emphasizes the concept that defective or inadequate repair subsequent to proteolytic or other damage in the lung is an important factor in emphysema. The validity of the inflammatory-repair hypothesis does not necessarily negate the protease-antiprotease theory, and destruction and aberrant repair in emphysema could contribute to the development of emphysema occurring as a result of protease-antiprotease imbalance.

A number of observations suggest that destruction of lung tissue is not sufficient on its own to account for the permanent destruction seen in emphysema:

1. Acute inflammation and tissue destruction observed in ARDS is an example of a massive neutrophil influx into the intra-alveolar space accompanied by destruction of respiratory tissue (Weiland et al. 1986). A large amount of NE is observed in the BAL of persons afflicted by this disease (Cochrane, 1983); however, emphysema is not an outcome of this disease.
2. Bronchoalveolar lavage fluid from lungs of patients with cystic fibrosis has greatly increased levels of elastase, but is not associated with the development of emphysema (Goldstein and Döring, 1986).
3. In experimental animals, an intensive accumulation of neutrophils is induced by deposition of immune complexes in the lung, and is accompanied by acute lung injury characterized by damaged al-

veolar epithelium and vascular endothelium (Ward et al. 1986); however, the lung appears to be normal within a week, without development of emphysema.

Lung injury acting in concert with defective repair may offer a plausible explanation accounting for the permanent destruction of lung parenchyma in emphysema. The inflammatory-repair hypothesis of emphysema has not been extensively studied in part because the mechanisms underlying lung development and repair are poorly understood to begin with. The reader is referred to a short review (McGowan, 1992) for a synopsis of current thinking in this area. The more convincing experiments which have supported a possible role of inflammatory-repair in emphysema are briefly outlined here.

The observation of fibrosis in centrilobular emphysematous lesions implies the involvement of repair in the progression of this disease. Permanent damage of lung tissue in emphysema suggests *per se* that there is a deficient repair process. The most convincing experimental evidence for the inflammatory-repair hypothesis arises from observations from animal models that experimental emphysema is induced through the inhibition of mature elastin formation by inhibiting tropoelastin cross-linking via:

1. β-aminoproprionitrile (BAPN) or other lathrytic agent-mediated inhibition of lysyl oxidase, a copper-dependent enzyme which mediates oxidative deamination of lysine residues, allowing for condensation to desmosine cross-links of tropoelastin (Kida and Thurlbeck, 1980) in growing animals, or in $CdCl_2$-treated animals (Niewoehner and Hoidal, 1982).
2. copper-deficient weaning of animals (O'Dell et al. 1978), acting possibly through lysyl oxidase*, as in (1),
3. genetic deficiency of lysyl oxidase in blotchy mice (Fisk and Kuhn, 1976)

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* N.B.- Copper is also a co-factor of the antioxidant enzyme, Cu-Zn superoxide dismutase and may arguably contribute to the development of experimental emphysema through this route as well.

Consistent with these observations was the finding that water-soluble component(s) of the gas-phase of cigarette smoke inhibit the formation of desmosine in the presence of tropoelastin in vitro (Laurent et al. 1983). Disrupted repair mechanisms distinct from elastin synthesis are also possible factors as all components of the lung parenchyma are affected by emphysema. Microscopic fibrosis in emphysematous lesions suggest the involvement of repair mechanisms consequent to damage. Elucidation of the mechanisms which distinguish development of emphysema as a consequence of lung injury as opposed to the development of fibrosis may provide some further insight into how permanent airspace enlargement arises in emphysema. An interesting analogy was made by Thurlbeck in a discussion regarding the possible effects of inflammation on the development of acute fibrosing alveolitis in contrast to emphysema:

> "Consider the skin. When exposed to sunlight in modest amounts, the inflammatory reaction is mild, resulting in erythema and subsequent pigmentation. If the skin is covered, it returns to its previous state... Recurrent or chronic exposure may result in degeneration of elastic tissue. If severe injury is induced in the skin, such as a wound or an abscess, there is destruction of tissue, production of fibrous tissue and a scar results." (Thurlbeck, 1986)

Determining the relationship between injury and repair in emphysema is an area which merits more extensive study.

Experimental models of emphysema.

Data regarding the pathogenesis of emphysema in humans are largely derived from epidemiological studies, which are complicated by population heterogeneity and differences in environment. With the exception of post-mortem studies, BAL, and incidentally collected biopsies, studies of emphysema in humans are also restricted to non-invasive techniques including physiological data, analysis of blood or

urine components (i.e. for connective tissue break-down), and more recently, high-resolution computed tomography. While studies based on these methods have provided much circumstantial evidence regarding the pathogenesis of emphysema, causative relationships cannot be extracted from these data.

Experimental models of emphysema are thus required to address the more important issue of causality. Models of emphysema have traditionally been based upon one of four approaches:

1. Intratracheal/ inhalation
2. Intravascular
3. Genetic mutants
4. Nutritional deficiency

More recently, transgenesis and gene-disruption via targeted homologous recombination have been employed, but have not yet been widely utilized in this field. The most accurate experimental models of emphysema are extended smoking models (e.g., Wright and Churg, 1990). The importance of smoking models however, lies primarily in their use as working models and does not directly address the issue of pathogenesis. An unavoidable area of concern is that extended smoking models produce emphysema in varying periods of time in different species. In humans, this is measured in decades, while in other species, it may be a matter of months. Among many possible factors which may contribute to inter-species variation in susceptibility to development of smoking-induced emphysema, are differences in proteolytic content per neutrophil. NE levels between different species for example, may vary by 20-fold*. Other factors are likely involved as well, and the possibility remains that the pathogenetic route involved may not be identical to that in humans.

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* Hamsters are reported to have 5% of NE per neutrophil relative to humans, and rats have 15-20% of NE (Glasgow et al. 1987).

In addition to the inescapable caveat that experimental animals are physiologically distinct from their human counterparts, a number of more specific and obvious difficulties have plagued most current models commonly used to study the pathogenesis of emphysema. The first demonstration of protease-induced emphysema was the observation by Gross in 1964 that intratracheal instillation of a plant-derived elastolytic cysteine protease, papain, led to airspace enlargement (experimental emphysema) in rats. NE was found to have a similar effect using this method (Janoff et al. 1977; Senior et al. 1977). Numerous studies have utilized the instillation technique as a standard means of assessing the ability of various agents in inducing emphysema and this technique has also been widely adopted as a means of assessing the efficacy of various protease inhibitors as potential therapeutic agents (e.g. Stone et al. 1992). The technical merit of this technique is that it allows the instillation of virtually any substance, allowing comparisons between the destructive potential of different substances. As one of the only means of inducing emphysema in experimental animals, the intratracheal instillation technique has become widely accepted; however, there are a number of shortcomings associated with this technique which must be seriously considered, but are generally overlooked:

1. Intratracheal instillation of destructive substances such as papain and NE is followed by hemorrhage, an inflammatory cell influx, and destruction of alveolar walls (Lucey et al. 1992). The possibility that the induced emphysema is a consequence of acute inflammation secondary to the instilled protease rather than a direct effect of the proteolytic activity of the instilled substance must be considered.
2. Intratracheal instillation does not allow for chronic manipulation; the induced emphysema arises from a single bolus of the tested substance while emphysema is a chronic disease, taking decades to develop in humans.
3. Intratracheal instillation may inaccurately portray the route through which these substances would act in vivo. For instance, proteases released from neutrophils may act predominantly from within the interstitial rather than the intra-alveolar space.

Together, these considerations raise the question of whether a substance shown to produce emphysema using this technique will induce similar sequelae with the chronic and low levels of exposure occuring in the actual development of the disease. The concentration of instilled substances used often has no physiological relevance. If a little does not work, more can be added. If airspace enlargement is then finally achieved, is there still physiological relevance? Evidently, while the intratracheal instillation method is effective in producing experimental emphysema, the interpretation of findings derived from these studies must be cautiously interpreted when extrapolating to the actual disease and in the worst scenario, the results can be misleading.

Genetic mutant models.

Genetic mouse mutants with airspace enlargement have supported some theories of emphysema pathogenesis. These are fortuitous models with congenital defects which may be developmentally related. Whether they share similar mechanisms with acquired emphysema in humans is debatable, depending upon the model:

Pallid mice are a model of human platelet storage pool disease. These mice have been shown by one group to have modestly reduced α1AT levels (Martorana et al. 1993), which this group suggests is the mechanism leading to enlarged airspaces in these mice. The pallid mutation is due to a mutation in a gene encoding a protein product of unknown function located on erythrocyte membranes and with homology to transglutaminase (White et al. 1992). It is not immediately apparent how this mutation would lead to decreased α1AT levels or whether there is truly any relationship between the decreased α1AT in this model and airspace enlargement.

The tight-skinned mouse is a model of human Marfan syndrome. Tight-skinned mice develop congenital airspace enlargement. The mutation is a duplication of the fibrillin-1 gene, which encodes an

ECM glycoprotein and is the mouse homolog of the human Marfan syndrome gene (Siracusa et al. 1996). This model suggests the potential importance of connective tissue repair/ assembly in emphysema but otherwise has no direct implications in our understanding of emphysema in humans.

The blotchy (mottled) mouse is a model of Menkes disease in humans. Spontaneous diffuse airspace enlargement occurs in this model. The genetic defect results in decreased expression of a protein required for proper copper transport; the human homolog of the defect is responsible for Menkes disease (Levinson et al. 1994). This model of experimental emphysema is analogous to the copper-deficient/ lysyl oxidase model of emphysema (see above).

The beige mouse is a model of human Chediak-Higashi syndrome and exhibits similar clinical symptoms (prolonged bleeding, reduced leukocyte chemotaxis, loss of natural killer cell function, giant lysosomes, etc.) which appear to stem from defective vesicular transport to and from the lysosome. NE and cathepsin G levels in these mice are reduced and possibly absent and this model has thus been used to determine the role of NE in emphysema. Endotoxin instillation produces airspace enlargement in normal mice but not in beige mice (Starcher and Williams, 1989), suggesting that neutrophil lysosomal components are important in endotoxin-induced experimental emphysema. The beige mutation is a deletion of a novel gene encoding a cytosolic protein of unknown function (Perou et al. 1996) and is the homolog of the human Chediak-Higashi gene. A number of biological features in addition to reduced NE and cathepsin G are seen in this genetic disorder (including abnormal alveolarization during development), and these defects should be considered in using this animal as a model of NE or cathepsin G deficiency.

The first model of genetically targeted increase in proteolytic activity demonstrating airspace enlargement resulted from expression of interstitial collagenase in the lungs of transgenic mice (D'Armiento et al. 1992). Transgenic models are a particularly suitable means of recapitulating chronic, low levels of increased protease expression, unlike intratracheal instillation, and will undoubtedly be

used in future studies of pathogenesis of this disease. Present difficulties with this technique include an inadequate understanding of the mechanisms leading to lung specific gene expression at appropriate levels and possible toxic effects of poorly controlled (mis)expression of transgenes in non-pulmonary tissues (see Chapter 2). Gene-disruption of suspected pathogenic factors with targeted homologous recombination, as has been demonstrated with MoE (see above), will be invaluable in determining the absolute relevance of these substances in producing emphysema (see Chapter 3).

Summary

The theory that NE is an important component in the pathogenesis of acquired emphysema is widely accepted but has not been adequately proven. The preceding analysis of the available literature regarding this subject supports a role for NE in the development of smoking-related emphysema, but it is also clear that definitive evidence for or against this hypothesis has yet to be shown. In consideration of the extensive amount of experimental work which has focused on the role of NE in emphysema and the current emphasis which is placed on developing inhibitors of NE, clarifying the role of NE in emphysema is a relevant issue which requires further study. In the following two chapters, attempts to produce experimental animal models to examine the likelihood that NE directly and significantly contributes to the development of emphysema are described.

Chapter 2: Development of a Model of Quantitative Variation in Neutrophil Elastase Expression.

Rationale and Hypotheses:

Previous studies examining the potential causative role of NE in the development of emphysema have relied upon intratracheal instillation models. NE and other substances were shown to have the potential to induce experimental emphysema using this method. As discussed in the previous chapter however, intratracheal instillation is a poor model of emphysema in humans. The advent of transgenesis has allowed a more accurate approach for assessing the potential of various endogenously expressed substances to induce emphysema and has been recently utilized for the first time in producing emphysema as a result of expression of interstitial collagenase in transgenic mice (D'Armiento et al. 1992). Much of the work regarding the pathogenesis of emphysema has focused on NE over the past two decades for reasons previously discussed (see Chapter 1). We have therefore proposed two models of transgenic NE expression to assess the feasibility of transgenesis as a means of studying the effect of increased NE burden in the lung. The following considerations formed the basis of our hypotheses:

The presumption that NE is important in the pathogenesis of emphysema implies that quantitative variation in NE levels would affect progression of the disease. In theory, variations in NE levels could explain why different individuals are differentially susceptible to the development of emphysema. A decreased level of endogenous NE inhibitors in susceptible individuals, as in α1AT-deficiency related emphysema, is one route through which relative NE activity can be increased; however, convincing evidence for such an association has not been demonstrated in the acquired form of emphysema. An increase in the number of neutrophils in the lungs of susceptible individuals, as discussed, is another possible route through which NE activity may increase. Previous data have suggested however, that numbers of neutrophils play only a limited role in mediating the differential development of emphysema (Chapter 1). Differences between individuals in levels of NE in each neutrophil resulting from differences in gene

expression is a third possibility which and would increase NE activity in the absence of either increased neutrophils or reduced antielastase levels. Although differences in NE levels are unlikely to account for all inter-individual variation in susceptibility to emphysema in smokers, it is a possible contributing factor. Transgenic models of NE expression have thus been proposed to address the following hypotheses and specific aims:

Hypotheses:

1. *NE is a potential pathogenic factor in the development of emphysema and therefore influences the development of the disease.*
2. *An increase in the endogenous level of NE protein expression would increase the level of NE activity, leading to increased susceptibility to development of emphysema.*

Specific Aims:

1. *Transgenesis is a feasible means of examining the effects of endogenously expressed proteases in producing emphysema and can be used to assess Hypotheses # 1 and 2.*
2. *Lung-directed or neutrophil-directed expression using the haptoglobin promoter or the NE 5' upstream region, respectively, in transgenic NE mice will increase NE burden in the lung and can be used to address Specific Aim # 1.*

The ability to address Hypotheses # 1 and 2 depends upon the feasibility of Specific Aims # 1 and 2. A number of potential technical difficulties are associated with the proposed models. Two issues of specific concern are: 1) the nature of the NE transgene and 2) the transcriptional regulatory elements to be used in directing expression of the transgene. The human NE gene was chosen as it can be readily distinguished from the endogenous mouse NE gene at both the protein and nucleic acid levels, was cloned and available for use, and is of direct significance to the human form of the disease. There is 77% se-

quence identity between mouse and human NE cDNA sequences. The NE gene rather than cDNA was chosen to maximize the efficiency of transgene expression (Brinster et al. 1988).

The HpNE transgene consists of a human haptoglobin promoter element fused to the NE gene. The haptoglobin promoter element in this construct is the same region used to direct expression of the previously described interstitial collagenase transgene (D'Armiento et al. 1992), consisting of the -1.05 kb of DNA immediately upstream of the human haptoglobin transcription initiation site. This is the only regulatory element which has been successfully used to drive expression of a protease-encoding transgene and causing experimental emphysema. Haptoglobin is normally expressed predominantly in adult liver in humans. In mice, haptoglobin is expressed in fetal and adult liver and at lower levels in the lung, skin, spleen, and kidney (D'Armiento et al. 1997). An early study had suggested that this region directs hepatocyte-specific expression, as indicated by in vitro transfection into HepG2 cells (Oliviero et al. 1987). Lung rather than liver-specific expression however, was observed with the 1.05 kb haptoglobin promoter driven interstitial collagenase construct and in a later study with a CAT transgene linked to the same promoter (D'Armiento et al. 1997). Low levels of transgene expression were also observed in the intestine, brain, and kidney. The exact cell type in the lung in which the transgene was expressed has not been identified. A possible explanation for the expression of human haptoglobin promoter directed transgenes to the lung is that although mouse haptoglobin is expressed at high levels in the liver, it is also normally expressed at lower levels in the lung, skin, and spleen (D'Armiento et al. 1997). This suggests that species-specific differences between trans-acting factors in lung and liver cells influences expression of the haptoglobin gene to a small degree, but does not account for the absence of expression in the liver, the predominant site of haptoglobin expression in both species.

In addition to the haptoglobin promoter, a number of other transcriptional regulatory elements are capable of directing transgene expression to the lung. These elements include but are not limited to the mouse surfactant associated protein-C (SP-C) promoter (Glasser et al. 1990) and the rat Clara cell 10-kDa protein (CC_{10}) promoter (Stripp et al. 1992). We chose to use the haptoglobin promoter primar-

ily because of its demonstrated ability to produce emphysema in the collagenase model. Although the haptoglobin transgene construct has been utilized successfully with interstitial collagenase, there have been no further studies examining the utility of this potentially important technique in studying other proteases. If the haptoglobin promoter can be used with NE or with other proteases, then the haptoglobin transgenesis technique can conceivably be used as a standard method for comparing the relative ability of different proteases to produce emphysema in lieu of intratracheal instillation.

A second means of increasing NE activity in the lung would be to increase its expression in its endogenous compartment (i.e., the promyelocyte) such that each neutrophil contains more NE in its granules. This approach would be a more physiologically accurate and rational approach than expression of NE directly in the lung. The disadvantages of this model are that 1) this particular model, if successful, could not be used to compare the effects of other, non-neutrophil primary lysosomal proteases, and 2) the mechanisms regulating expression of NE or of other primary granule proteins are poorly understood (see Part II) and transgenic expression of NE in the promyelocyte may therefore not be a feasible undertaking at the present time. Many studies have described the ability of DNA immediately flanking the 5' end of a gene to direct tissue-specific expression as a tramsgene, often at low levels relative to endogenous expression of that gene; however, this is not always the case, as seen with the haptoglobin promoter. The issue of tissue-specific expression is, of course, a complex issue, and in instances where no precedent studies have been made, a crude practical approach would be to use an arbitrarily large 5' flanking region to guide expression of the gene of interest. The PB74 genomic clone was the largest genomic clone of the human NE gene available at the time when these experiments were designed, although much larger genomic clones have since become available. PB74 consists of the complete NE gene coding region with ~1.4 kb and ~0.7 kb of 5' and 3' flanking DNA, respectively. Two observations supported our decision to use this clone as a transgene (HNE) to over-express NE in its endogenous compartment:

1. A functional myeloid-specific promoter of NE had been identified using *in vitro* assays in cell lines and was observed to occur within 153 bp of the transcriptional initiation site. An enhancer of transcription was also observed at ~1 kb 5' of the transcriptional initiation site (Chapter 4).
2. Previous studies have demonstrated that adjacent 5' elements located from less than 100 bp to greater than 10 kb may be involved in the regulation of gene expression at a locus (see Chapter 4). Often, transgenic studies identify elements within 1-2 kb of the transcription initiation site that are able to mediate varying levels and accuracy of gene expression in transgenic animals.

As the mouse and human NE promoters share common transcription factor binding sequences (Oelgeschläger et al. 1996; also see Chapter 4), and in consideration of the general conservation of tissue-specific gene expression mechanisms between mammals, production of HNE transgenic mice might demonstrate the ability of the 1.4 kb 5' flanking region of NE to direct expression of NE in vivo, and may thus also provide insight towards the mechanisms governing transcription of the NE gene (Part II).

Methods*

Preparation of DNA constructs†

HpNE is the 1.05 kb haptoglobin promoter fused to the human NE gene (ELA2) and has been used to drive expression of the interstitial collagenase gene in the lung (above). HpNE was prepared as follows: The 1.05 kb haptoglobin promoter was amplified by PCR from human genomic DNA using the BGR3 and BGR4 primers (see Appendix), incorporating restriction sites for BamHI and EcoRI at the 5' and 3' ends of the fragment, respectively (Fig 2.1). The size of the amplified fragment was as expected, appearing as a ~1.1 kb fragment on agarose gel electrophoresis. This fragment was subcloned into the BamHI/ EcoRI sites of pBluescript II SK +/- (pBS) to form pHp(BS) and the 5' ~250 bp was confirmed by DNA sequencing to match the published sequence. Genomic NE was derived from the 6 kb PB74 genomic clone of ELA2 (from R.G.Crystal, NHLBI, NIH) as follows: The PB74 insert was subcloned into three fragments to facilitate PCR amplification of the 5' fragment (Fig 2.1). The PB74 insert was digested to completion with BamHI/ EcoRI or Bam HI alone. A ~2 kb 5' EcoRI to BamHI fragment containing exon I was

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* All the experimental, technical, preparatory, and research work presented in this thesis were conducted entirely by the author except where indicated in the footnotes or text.

† Plasmid minipreps, mid-sized preps, polymerase chain reactions, bacterial transformations, Southern, Northern, and dot-blotting, restriction digests, DNA or RNA radiolabeling, ligations, and other simple enzymatic and general molecular biology manipulations are according to standard procedures (Sambrook et al., 1989) except where indicated. All sequencing was performed with the Sequenase 3.0 kit (USBiochemicals) according to the manufacturer's directions, in the vectors mentioned, unless otherwise stated. All DNA was purified via PEG precipitation prior to sequencing.

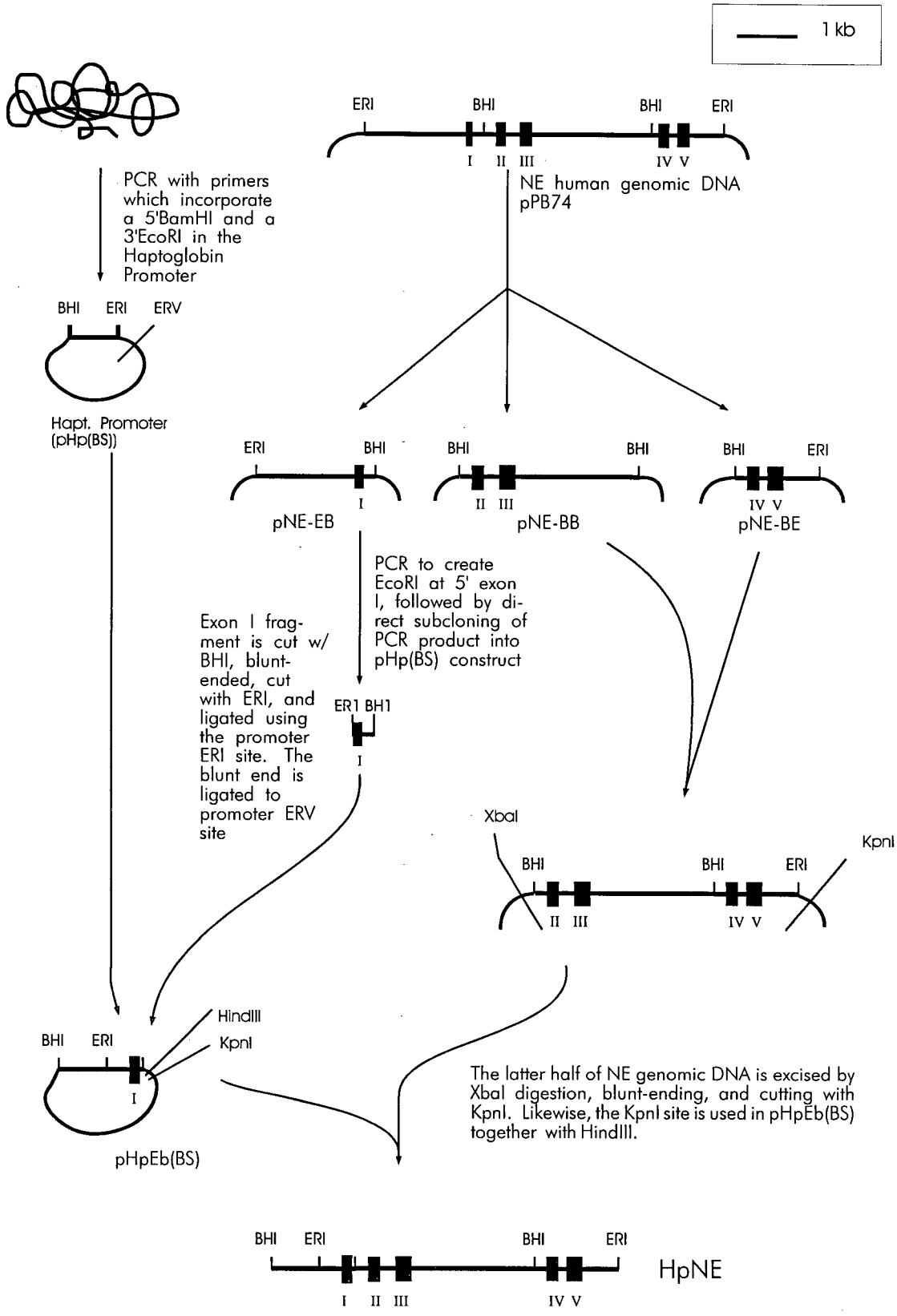
1 kb
ERI
BHI
BHI
ERI
I II III
IV V
NE human genomic DNA
pPB74
PCR with primers which incorporate a 5'BamHI and a 3'EcoRI in the Haptoglobin Promoter
BHI ERI ERV
Hapt. Promoter (pHp(BS))
ERI BHI
I
pNE-EB
BHI BHI
II III
pNE-BB
BHI ERI
IV V
pNE-BE
PCR to create EcoRI at 5' exon I, followed by direct subcloning of PCR product into pHp(BS) construct
Exon I fragment is cut w/ BHI, blunt-ended, cut with ERI, and ligated using the promoter ERI site. The blunt end is ligated to promoter ERV site
ER1 BH1
I
XbaI
KpnI
BHI
BHI
ERI
II III
IV V
HindIII
KpnI
BHI ERI
I
pHpEb(BS)
The latter half of NE genomic DNA is excised by XbaI digestion, blunt-ending, and cutting with KpnI. Likewise, the KpnI site is used in pHpEb(BS) together with HindIII.
BHI ERI
BHI
ERI
I II III
IV V
HpNE

Figure 2.1- Summary of HpNE transgene construction.
The haptoglobin promoter was amplified from genomic DNA and subcloned. The ELA2 5′ coding region was derived from PCR-amplification of the pNE-EB subclone, from the transcription initiation site to the 3′ end of the subclone. The amplified-5′ end fragment was ligated to the haptoglobin promoter construct, and the coding region was reconstructed as shown. Horizontal lines depict non-coding DNA; filled boxes represent exons, and relative locations of the restriction sites used to construct the transgene (BHI, BamHI; ERI, EcoRI; ERV, EcoRV) are indicated as shown. Refer to text or figure for further detail.

directionally subcloned into the BamHI/ EcoRI sites of pBS; the adjacent 2.5 kb BamHI to BamHI fragment containing exons II and III was subcloned into BamHI-digested, calf intestinal alkaline phosphatase (CIAP)-treated pBS and the orientation deduced from restriction enzyme digestion. The 1.5 kb 3' BamHI to EcoRI fragment, containing exons IV and V was directionally subcloned into the same sites in pBS to create pNE-BE. PCR amplification with the BGR1 and T3 primer-pair (see Appendix) was used to isolate a region of the 5' exon I-containing subclone from the first nucleotide in exon I (BGR1) to the 3' end of the construct (T3 site in pBS), introducing an EcoRI site (via BGR1) at the 5' end to facilitate ligation to the haptoglobin promoter. The fragment was digested with BamHI, the BamHI site was filled (blunt-ended), and the fragment directionally subcloned into the EcoRI/ EcoRV site of pHp(BS) to form pHpEb(BS). The T3 and T7 primers (flanking the pBS polylinker region) were used to sequence the 5' and 3' ends of the construct to ensure integrity of the blunt-end ligation. The 2.5 kb BamHI to BamHI fragment containing exons II and III was non-directionally subcloned into the BamHI site of pNE-BE; a subclone containing the 2.5 kb fragment in the correct orientation was selected using restriction analysis. The entire insert, containing exons II to V, was excised using XbaI / KpnI and directionally subcloned into the HindIII-KpnI site of pHpEb(BS) in the correct transcriptional orientation, resulting in the pHpNE construct in which the haptoglobin 1.05 kb promoter is fused to the immediate 5' end of the NE gene. The HpNE insert was excised from pHpNE using NotI / SalI, and the entire insert was released as a single fragment for use in producing HpNE transgenic mice. The insert was separated from the vector via electrophoresis in 0.6% TBE agarose gels and purified with GeneClean II (Bio 101) according to the manufacturer's directions and with special precautions to prevent shearing of large DNA fragments. After the integrity and concentration of the DNA was determined by agarose gel electrophoresis/ ethidium bromide staining with size/ quantitative DNA markers and UV spectrophotometry, the constructs were eluted into TE buffer (5 mM Tris-HCl [pH 7.4]: 0.1 M EDTA) and diluted to 1.5 ng/μL in the same buffer for microinjection.

HNE is the ~6 kb EcoRI clone of the human NE gene (gene symbol, ELA2; Fig 2.2A, page 55) encompassing all 5 exons and including ~1.5 kb of 5' adjacent DNA and ~1 kb of 3' adjacent DNA (Takahashi et al. 1988b). Restriction enzyme digests with EcoRI and BamHI were used to confirm the integrity of the insert. The HNE insert is removed from PB74 using EcoRI and purified in preparation for microinjection as described above for HpNE.

The genomic probes used for Southern hybridization of transgenic animal DNA were the human NE cDNA and a 700 bp Pst I genomic fragment of PB74 contained within the BamHI fragment of the NE gene and specific for the human transgene DNA (Fig 2.2A). This latter probe was determined not to contain repetitive elements using a reverse Southern blot (hybridization of radiolabeled total mouse genomic DNA to Southern blotted probe fragment) and is specific for the human neutrophil elastase gene (hybridization to a single band on BamHI cut human genomic Southern blot but not with normal mouse DNA) (data not shown). The human cDNA sequence was shown to hybridize uniquely to the human neutrophil elastase gene and does not cross-hybridize to mouse DNA under stringent conditions (65 C, 0.1 x SSC, 0.5% SDS) (not shown).

Transgenesis

Egg preparation and microinjection. Fertilized eggs used for microinjection were derived from superovulated female BALB/c mice: Four units of Gestyl (pregnant mares' serum, mimicking FSH) were administered to 3 to 5 week old female BALB/c mice via intraperitoneal injection at 4 p.m. three days prior to egg isolation, and 4 U Pregnyl (human chorionic gonadotropin, mimicking LH) were administered via the same route at 12 to 1 p.m. the day before isolation and these females were immediately mated with CD-1 males. All mice were kept at a 6 a.m. to 6 p.m. light/ dark cycle. Eggs were isolated the following day from the oviducts of successfully mated females in 37°C $BMOC_2$ medium in the presence of ~0.5 mg/ml hyaluronidase. The eggs were disaggregated, rinsed twice in $BMOC_2$ medium without hyaluronidase and placed at 37°C, 5% CO_2 in the same medium for microinjection. The appropriate

transgenic DNA at 1.5 ng/μL in TE (5 mM Tris-HCl [pH 7.4]: 0.1 M EDTA) buffer (above) was micro-injected into the pronucleus of fertilized cells using a standard technique*.

Egg transfer and identification of transgenic mice. Pseudopregnant female mice were prepared by mating healthy, mature (>8 week-old) CD-1 females in natural estrus with vasectomized males the day before egg transfer. An incision through the skin and body wall was made along the right or left lower-dorsal region of the mouse through which the oviduct was accessed. A small tear was made in the bursa and 10 to 20 visibly live (intact nuclei), fertilized, microinjected eggs in $BMOC_2$ medium were transferred into the right or left infundibula of anesthetized pseudopregnant females.

Dot blots and Southern blotting were used to identify transgenic HpNE or HNE pups. Approximately half a centimeter of tail was removed and incubated in DNA digestion buffer (50 mM EDTA, 50 mM Tris-Cl pH 8, 0.5% SDS, 0.5 mg/ml proteinase K) at 55° C for 8 to 12 hours; solid components were removed by centrifugation, excess proteins and lipids were extracted with phenol and chloroform, and the genomic DNA was ethanol precipitated, washed in 70% ethanol and quantitated using UV spectrophotometry. Ten micrograms of DNA were dot- or Southern-blotted onto nylon membrane and positive transgenic mice were identified using a suitable ^{32}P-radiolabelled human NE genomic DNA probe (see Results and Discussion) under stringent conditions. Transgene copy numbers were roughly determined through densitometric comparison of quantitated transgenic mouse-tail DNA with a series of increasing transgene construct DNA diluted into a similar quantity of non-transgenic mouse tail DNA prepared in the same method. Transgene integrity was confirmed with Southern analysis.

Immunohistochemistry

Immunohistochemical detection of human NE in peripheral mouse or human blood and bone marrow smears was performed as follows: Air-dried blood smears were fixed with 100% methanol for 1

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* Microinjection of DNA into oocyte pronuclei was kindly performed by Dr.S.Porter.

minute, rinsed in 1x TBS for 30 seconds, incubated with 1:50 polyclonal rabbit anti-human NE antibody (Elastin Products Company) in PBS/ 3% BSA for 2 hours in a humid incubation chamber, rinsed in 1x TBS for 1 minute, incubated with 1:200 alkaline-phosphatase conjugated, affinity purified goat anti-rabbit IgG (Vector Laboratories) for 45 minutes in a humid chamber, and rinsed in 1x TBS for 3 minutes. Alkaline phosphatase activity is detected with the Napthol AS-TR Phosphate substrate/ Fast Red RC detection system (Sigma Chemical Co.) which produces an insoluble red precipitate and the smears were counterstained with toluidine blue. The primary antibody was specific for human NE and did not cross-react with mouse NE (see Results, Fig 2.3A).

Isolation of RNA and Northern blot hybridization

Total cellular RNA was isolated from bone marrow flushed from mouse femurs with 1x PBS using a 23-gauge needle and from U-937 cells cultured in RPMI 1640 supplemented with 10% fetal calf serum. Bone marrow cell smears were made, air-dried, fixed in methanol and stained with haematoxylin and eosin (H & E) to determine the presence of cells. Total RNA was isolated with Trizol Reagent (GibcoBRL) according to the manufacturer's directions or with the single-step guanidine method. RNA integrity was confirmed with ethidium bromide staining and visualization of the 28S and 18S rRNA bands. Fifteen micrograms of intact RNA per sample were denatured in 50% formamide, 2.2 M formaldehyde and 20 mM sodium phosphate, pH 7.6, and separated in a 1% agarose-formaldehyde gel. RNA was transferred onto Nytran Plus membrane (Schleicher & Schuell) and hybridized with ^{32}P-dCTP radiolabeled mouse or human NE cDNA as indicated.

Results and Discussion

The HpNE Model

A prospective aim of this study was to develop a model of endogenous NE over-expression (Specific Aims # 1 and 2). By using the lung-specific haptoglobin promoter construct, the suitability of the haptoglobin-construction as a general technique for exploring the effects of lung-specific protease expression can be evaluated. The HpNE transgene, whose construction is detailed here, consists of the 1.05 kb 5' proximal flanking region of the haptoglobin gene, previously used to drive lung-specific expression of transgenic collagenase (D'Armiento et al. 1992), ligated to the transcription initiation site (+1) of the human neutrophil elastase gene. Thirty-six mouse pups were obtained (Table 2.1). Dot blot hybridization of tail-derived DNA with radiolabeled HNE cDNA in the presence of positive control DNA demonstrated that only one of these mice carried transgenic DNA and Southern analysis of various restriction enzymes probed Southerns limited the possibility of integration to a single-copy, rearranged transgene (not shown). The rearranged transgene would not be expected to be expressed in a functional form and

Table 2.1- Frequency of transgenic progeny

Transgene	sample size	transgenic progeny (%)
HpNE	36	0
HNE	35	14
UPMT	32	16
5PT	49	13

Abbreviations: HpNE and HNE are described in text. UPMT and 5PT are tyrosinase-encoding transgenes encoding the human tyrosinase cDNA produced in our laboratory (Porter and Meyer, 1994).

perhaps not at all. Northern blot analysis of lung RNA from homozygous mice confirmed the absence of transgenic human NE RNA in the lungs of these animals (not shown), indicating that the rearranged transgene was not expressed.

The frequency of HpNE transgenic progeny was in distinct contrast to the expected frequency of derivation of transgenic lines in our lab (Table 2.1). Of particular relevance is the observation that HNE transgenic animals were prepared at the same period of time and with similar techniques, materials, and personnel as for the HpNE experiments, and exhibited a transgene integration frequency of 14%. Other transgene constructs used in our laboratory had similar rates of transgene integration frequencies. A simple statistical analysis based on these previous observations, assuming that the frequency of transgene integration is a normally distributed event, suggests that there would be a 99% probability that a minimum of 3 (8.2 %) transgenic pups would be found within 36 pups derived from microinjected oocytes. Since 0/36 transgenic pups were obtained, the null hypothesis (no difference in transgenesis frequency) is rejected, and this data therefore strongly suggests that incorporation and expression of the HpNE transgene is detrimental to the viability of the developing embryo. This observation contrasts with the successful use of the 1.05 kb haptoglobin promoter region to drive expression of the interstitial collagenase transgene (18% transgenesis frequency, n=79 liveborn mice). This discrepancy can in theory be attributed to a more toxic effect of ectopic expression of NE in comparison to interstitial collagenase in the same tissues, perhaps due to the more destructive proteolytic profile of NE (Chapter 1). ECM-degrading proteases have been observed to be involved in the process of tissue invasion in the implantation of the embryo in the uterine stroma (Harvey et al. 1995). It is therefore possible that if NE were expressed during this stage of development, normal embryonic implantation would be disrupted. Ectopic expression of NE at later periods in embryonic development may also presumably have detrimental effects on viability of the embryo. This interpretation is supported by the observation that expression of NE in tissue culture was not possible (D.Jenne, personal communication; Max Plank Institute, Münich); only inactive mutant forms of the transgenic protein can be expressed. A third possibility is that expression of

NE in the lung itself is toxic to the developing embryo. The precise cause of lethality in HpNE embryos and the stage at which this lethality occurred was not specifically further examined as human haptoglobin promoter-driven expression to the lungs of mice is an artifact of improper ectopic transcriptional regulation and thus not of further apparent academic or practical interest.

More importantly, by demonstrating the toxicity of HpNE transgene expression in mice, it can be concluded that the 1.05 kb haptoglobin promoter construction is not suitable for use as a standard regulatory element that can be used to compare and assess the effects of different proteases in the lung, as suggested in Specific Aims # 1 and 2. Given the emphasis that is placed on the protease-antiprotease hypothesis, a coordinated effort towards development of a transgenic model for studying the role of NE and other proteases in the development of emphysema would be of benefit in furthering our understanding of the specific nature of protease-antiprotease interaction in the development of emphysema and could thus eventually aid in the design of rational therapy for the disease. A gene regulatory element with more restrictive lung-specific expression would be necessary in any future attempts to create a similar model. The SP-C promoter is one of several conceivable candidates for mediating this sort of expression; however, as with the haptoglobin construct, the only accurate means of determining their suitability for this use is through empirical observation, as even minute levels of undesired expression could presumably be toxic.

A further potential criticism of lung-specific expression in transgenic animals as a model of emphysema is that constitutive expression throughout development may lead to congenital/ developmental defects in the lung through mechanisms unrelated to those involved in the pathogenesis of acquired emphysema, a disease which develops in the fully developed lungs of adult humans. A possible means of circumventing this problem would be to use an adult-specific or an inducible model of lung-specific expression. This would also minimize the possible detrimental effects of expression during development as observed with the HpNE mice. Another means of avoiding this problem is to express the protease in its

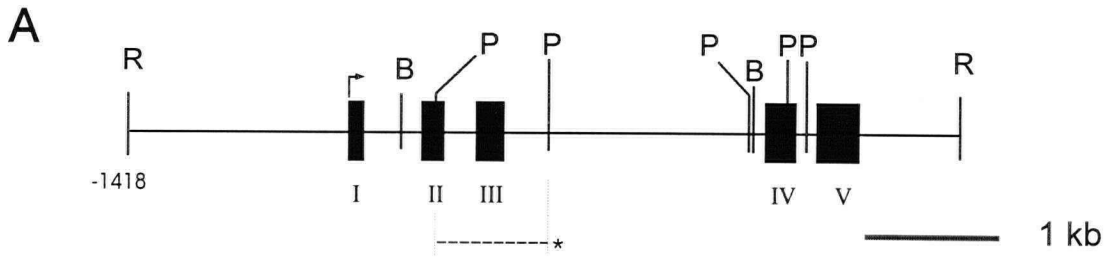
normal compartment, i.e., the promyelocyte. The HNE model (below) was proposed to address this possibility.

The HNE model

To address the hypothesis that genetic differences in protease levels may be partially responsible for differences in susceptibility to the development of emphysema, a genetic model of endogenous NE over-expression was proposed. In contrast to the HpNE construct, the HNE construct was designed with the intention of increasing NE specifically in its endogenous compartment, the promyelocyte, through the integration of an additional (human) NE transgene(s). The susceptibility of mice with increased NE (mouse + human) would be compared to non-transgenic mice. The HNE transgene encompasses the entire NE gene, including 1.4 kb of 5' flanking DNA and ~0.7 kb of 3' flanking DNA. DNA from 35 mouse pups derived from HNE transgene microinjected oocytes were produced; dot blot analysis of tail-DNA from these pups identified five animals carrying transgenic DNA (HNE1 to 5) (not shown). These animals were physically similar to their non-transgenic litter-mates. The HNE5 mouse was dead when examined shortly after birth; it cannot be determined whether the animal died pre- or post-partum. Southern analysis of BamHI digested DNA from HNE 1 to 5 with human NE cDNA confirmed the presence of transgenic DNA and demonstrated the presence of multiple copies of the transgene (Fig 2.2B). The size of the hybridizing bands were as expected for all five lines, suggesting that the complete transgenes had successfully integrated intact. From 2 to >10 HNE transgene copies per line were determined through comparison with quantitated transgene DNA controls (Fig 2.2B and not shown).

To determine the presence of transgenic protein in neutrophils from these mouse lines, immunohistochemistry of transgenic peripheral blood was performed on peripheral blood smears from mice derived from each of the transgenic lines. Neutrophils from HNE1 to HNE5 did not exhibit any detectable transgenic HNE protein in contrast to readily detectable levels of human NE in human peripheral blood

neutrophils in control slides (Fig 2.3A). To determine whether absence of transgenic NE protein stemmed from lack of HNE transcription, Northern analyses were performed: H&E-stained bone marrow cells flushed from the transgenic mouse femurs demonstrated a predominantly immature hematopoietic cell population. Intact RNA was successfully isolated; however, radiolabeled human NE cDNA probes did not hybridize to total RNA derived from transgenic mouse marrow, whereas the probe hybridized readily to positive human controls (Fig 2.3B). Examination of a constitutively expressed gene to normalize the control with the sample RNA was not feasible since two different species were used. To address the possibility that lack of detectable transgene RNA was due to insufficient total mouse marrow derived mRNA, a reciprocal analysis was made in which the same blots were probed with mouse NE, whereupon mouse NE RNA was readily detected, while the human sample did not hybridize, indicating that sufficient levels of endogenous NE transcripts were present for detection on the Northern blot and that inability to detect transgenic HNE mRNA therefore resulted from undetectably low levels of transcription. These observations indicate that lack of transgenic NE protein in HNE transgenic mice was due to lack of transcription of the integrated HNE transgenes in HNE 1 to 5, implying that any transcriptional regulatory elements encompassed within the PB74 region represented by the transgene, including the previously described promoter and enhancer regions of the NE gene, were not sufficient to induce expression of the NE gene in the bone marrow cells of mice in vivo.



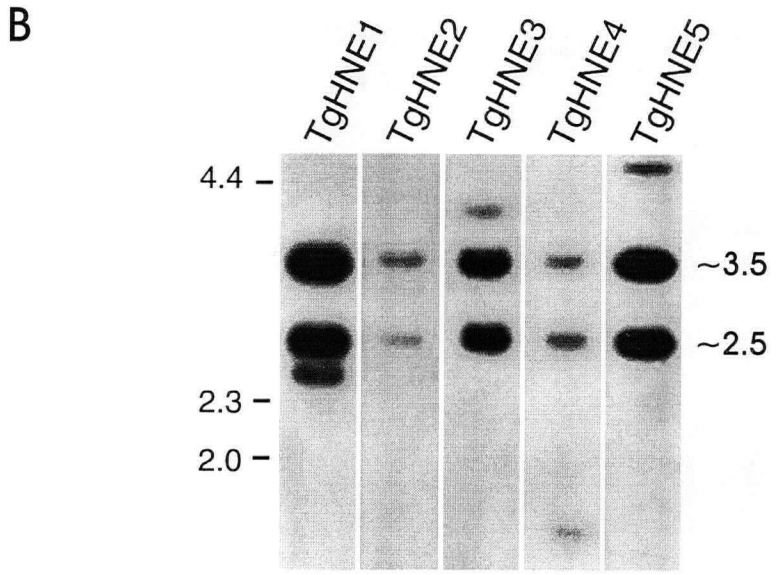


Figure 2.2- HNE transgenic mice.
(A) Scale restriction map of the HNE transgene (PB74) showing location of sites (thin vertical lines) used in generating the construct and identification of transgenic animals. B, BamHI, P, Pst I, R, EcoRI. The thin horizontal line depicts non-coding genomic DNA and the filled boxes show the relative location of the exons. An internal PstI fragment subcloned for use as a probe is shown below the map as indicated. (B) Southern blot analysis of transgenic founder lines. BamHI digested mouse-tail derived DNA was hybridized with an ELA2 cDNA probe. The locations of the expected 3.5 kb band that would arise from hybridization of exons 1, 4, and 5 to the 5' and 3' fragment (integrated into a head-to-tail tandem array) and of the 2.5 kb internal BamHI fragment arising from hybridization of exons 2 and 3, are indicated on the right; the locations of the DNA markers used are marked on the left. Additional bands are expected from the ends of the integrated transgene array, and are seen in each lane.

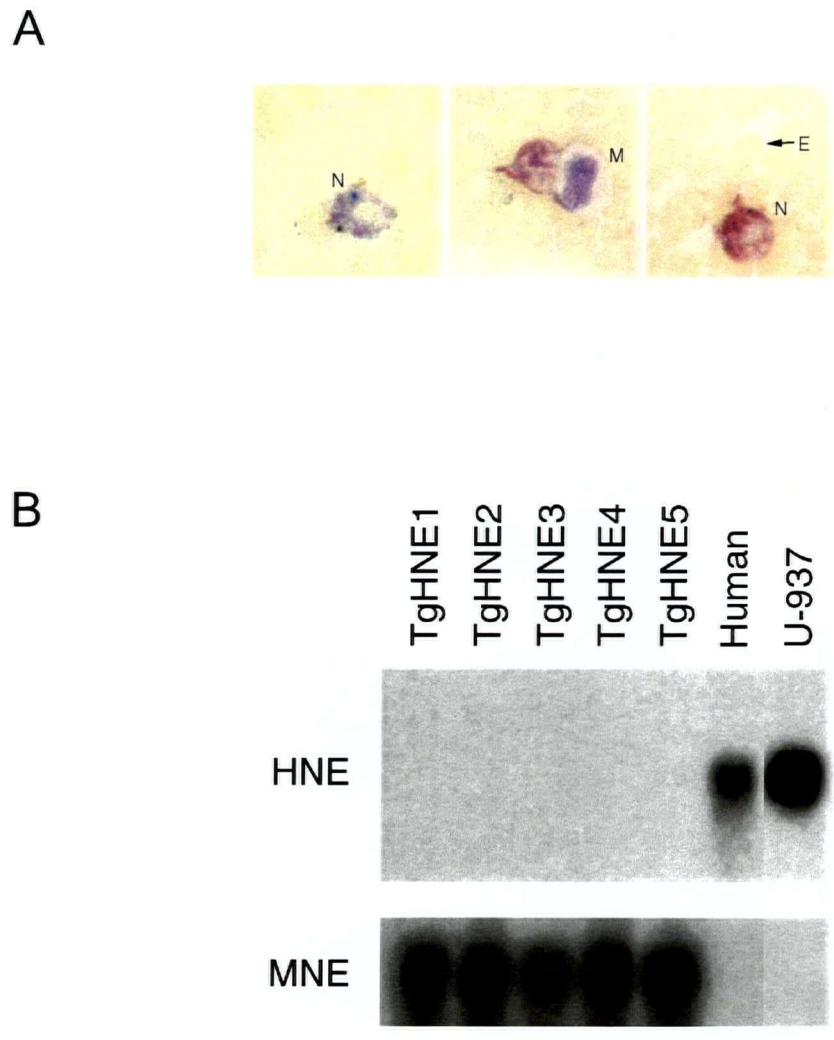


Figure 2.3- HNE transgene expression
(A) TgHNE3 mouse (left) and human (middle and right) peripheral blood smears were analyzed with anti-human neutrophil elastase-specific polyclonal antibody, visualized with the Sigma Fast Red RC detection system, which produces an insoluble red precipitate, and lightly counter-stained with toluidine blue. No red precipitate was observed in blood leukocytes from TgHNE3 (left) or TgHNE 1,2,4,5, or in non-transgenic mice (not shown). In human control smears, the red precipitate was abundant in neutrophils and obscures the light toluidine blue staining; N, neutrophil; M, monocyte; E, erythrocyte. (B) Northern analysis of bone marrow-derived RNA from transgenic TgHNE 1 to 5 lines, from human marrow, or from U-937 early myeloid cells with a radiolabelled human NE cDNA probe and mouse NE probe, as indicated.

Summary

Although the HpNE and HNE constructions did not allow examination of the effects of NE in the lung as hypothesized, several issues have been raised by these experiments with respect to the feasibility of using transgenesis in the creation of genetic models of protease expression. Firstly, ectopic protease expression in the lung may be lethal; this could possibly result from leaky expression in non-pulmonary tissues or from a direct toxic effect of expression in the lung in utero and potentially after birth. Identification of transcriptional regulatory elements capable of driving expression only in the adult or dependent upon addition of an inducing agent would be beneficial. Expression of proteases such as NE in their endogenous compartment is also potentially problematic when the regulation of tissue-specific expression has not been adequately studied. The use of transgenesis in the study of lung function and disease is relatively new (reviewed in Ho Y.-S., 1994), but has presented the possibility of overcoming numerous previous limitations in the study of lung disease. There appear to be several technical hurdles which must be overcome however, before this approach can be commonly employed in the study of protease action in lung disease. Transgenesis has been used to develop models of antiprotease over-expression in the lung; while such studies have important potential clinical applications, antiprotease over-expression is of limited use in defining pathogenic mechanisms of disease for the reasons discussed. With the exception of the collagenase transgenic mouse, reports of successful protease over-expression in the study of lung disease have not been described to date.

Chapter 3: Towards Development of a Model of Neutrophil Elastase Deficiency: Cloning the Mouse NE Gene.

Rationale and Hypotheses:

A threshold level of α1AT appears to be necessary for protection against adverse proteolytic destruction of lung tissue in emphysema. It is similarly possible that a critical level of proteolytic activity conferred by NE or some other protease is *necessary* to result in the destruction of lung tissue that is characteristic of emphysema. Although the *sufficiency* of a substance to induce emphysema in experimental models may demonstrate the potential for that substance to produce the disease, it provides no indication as to the relative role of that substance in the pathogenesis of emphysema, or even whether the substance is *necessary* in the development of this disease. Given the emphasis which has been placed on NE in emphysema, an important advance in the current understanding of emphysema would be to assess the necessity or relative contribution of NE to the development of this disease. An ideal model for examining this issue would be, in theory, to study lung function in a naturally occurring model of NE deficiency in humans; however, genetic NE-deficiency in humans or animals has not been reported. The Chediak-Higashi syndrome is the closest known model of NE deficiency in humans and is characterized by a number of defects of the neutrophil, including neutropenia, impaired chemotaxis, and deficient degranulation. There are however, no reported epidemiological studies correlating lung function and cigarette smoking in individuals with this disease. A mouse model of Chediak-Higashi syndrome (beige mouse, see page 36) has been identified, and experiments with these animals appear to support the role of NE in the development of emphysema. The large number of associated defects in this model however, limits the possible interpretations that can be derived (Chapter 1).

The advent of gene knock-out technology in mammals permits the production of a NE-deficient animal model. The following specific hypotheses were therefore proposed:

1. *NE is a potential pathogenic factor in the development of emphysema and therefore influences the development of this disease (equivalent to Chapter 2, Hypothesis #1; page 40)*
2. *A decrease in the endogenous level of NE protein would decrease the level of NE activity, leading to a decreased susceptibility to development of emphysema.*
3. *Disruption of the NE gene via targeted homologous recombination in mice and induction of experimental emphysema in comparison to wild-type control animals and to MoE deficient mice will demonstrate the necessity of NE in the development of emphysema and the relative contribution of NE to this disease, respectively.*

Towards this end, we had proposed the construction of NE-deficient mice. We suggested that susceptibility to the development of smoke-induced emphysema in these mice in comparison to wild-type mice with normal levels of NE would demonstrate the relative importance of NE in the pathogenesis of smoke-induced emphysema. Several outcomes are possible from such a study:

1. The model is not feasible, i.e., homozygous NE-deficiency is lethal *in utero*. Given our present understanding of the primary biological role of NE as an antimicrobial protease, this outcome appears to be unlikely. Increased susceptibility to infection would be expected; however, sterile animal rearing should prevent infection.
2. NE-deficiency renders the animal less susceptible to experimentally induced emphysema. This would suggest a likely role of the protease in the pathogenesis of the disease. How much this effect contributes to the overall susceptibility to development of the disease would still need to be considered, and this could only be done on a comparative basis with other suspected agents of disease using a similar experimental design. For instance, direct comparison with MoE(-/-)

mice (above) would indicate the relative contribution of each protease to the development of experimental emphysema.

3. NE-deficiency has no effect on experimentally induced emphysema relative to controls. In this scenario, the hypothesized role of NE in inducing emphysema would be largely discredited. In consideration of the large emphasis that has been placed on the putative role of NE in emphysema, this finding would substantially affect current thinking in this area.

To produce a NE knock-out mouse, it is first necessary to clone genomic mouse NE. In this chapter, cloning of the mouse NE gene and partial construction of the replacement construct is described. While this work was underway, we were notified that a laboratory in St.Louis had already successfully obtained homologous recombinants with an inactivated NE gene in ES cells, compelling us to relinquish the project at that stage. As such, complete localization and confirmation of all the mouse NE exons and restriction sites and completion of the gene targeting protocol were completed only in part and the data provided in this section is not complete. The NE knock-out mouse was reported three years later (Belaaouaj et al. 1998), reporting the importance of NE in host defense against gram-negative bacteria. Cigarette smoking experiments have not yet been described for this model; however, the results should be reported imminently. As the outcome of NE-deficient mouse cigarette smoking experiments were not reported at the time this thesis was written, no further speculations can be made here. As mentioned in the previous discussion, the results of smoking experiments on NE-deficient mice will likely have important implications with regards to our present understanding of the disease. My work in partial cloning of the mouse genomic NE gene and the outline for construction of the gene targeting construct is briefly described here:

Methods

Cloning mouse genomic NE (Ela2)

Mouse NE (gene symbol, Ela2) is the proposed region to be targeted and requires the mouse NE gene. To clone Ela2, the 129SV mouse genomic library (Stratagene, La Jolla, CA), which is derived from 9-22 kb Sau3AI partially digested genomic DNA cloned into the XhoI site of Lambda Fix II vectors, was used. The library was diluted and plated according to the manufacturer's recommendations to ~4 x 10^4 pfu/ plate. Plaques were transferred to nylon membranes (Schleiser and Schull), denatured, neutralized, and rinsed according to the manufacturer's directions. Screening was performed with ^{32}P-radiolabeled mouse NE cDNA probes (M.Naruto, Osaka) on a total of two sets of ~1.5 x 10^6 plaques. Positive plaques were isolated and confirmed through secondary and tertiary screening using similar procedures.

Characterization of clones

Lambda phage DNA was isolated from plaques with LambdaSorb phage adsorbent (Promega) according to the manufacturer's indications (plate-lysis method). The size of DNA inserts from lambda phage was estimated with agarose gel electrophoresis of the intact insert and also deduced through sizes of sub-bands. Restriction enzyme sites for BamHI, EcoRI, HindIII, and XbaI were mapped through hybridization of partially digested clone DNA: The Lambda Fix II clone inserts were digested with NotI to isolate the clone from lambda phage vector arms. 0.1U/μL of each restriction enzyme was used to digest 5 μg of clone DNA for an optimized time-period (to obtain the greatest range of restriction fragment lengths), separated by electrophoresis in 0.7% agarose, Southern blotted, probed with T4 polynucleotide kinase (PNK)-mediated γ-^{32}P ATP radiolabeled T3 and T7 oligonucleotides, and washed with 6x SSPE/0.1% SDS at 42°C. The location of restriction sites used for subcloning were verified with restriction enzyme digestions with a panel of restriction enzymes and the appropriate restriction sites were used to clone pmNEg1, 2, and 3 (above and Fig 3.1A). Location of mapped sites were partially confirmed through determination of restriction fragment sizes by agarose gel electrophoresis. The relative locations of the ex-

ons and introns within the clones were determined through PCR-mediated amplification of intronic DNA sequences with mouse NE cDNA-derived primer pairs as indicated in Figure 3.1A. Sequencing with Sequenase v.2 (USBiochemicals) was used to confirm the presence of exons I and II.

Preparation of constructs

Clone A was subcloned into pBS as pmNEg1, pmNEg2, pmNEg3, and pmNEg4, to facilitate further analysis (Fig 3.1A). The plasmid pmNEg1 is a ~5.5 kb Not I/ Eco RI fragment at the T7 end of Clone A; pmNEg2 is a ~5.5 kb EcoRI fragment; pmNEg3 is a ~2 kb NotI/ EcoRI fragment at the T3 end of Clone A; pmNEg4 is a ~2.6 kb HindIII/ XbaI fragment derived from pmNEg1. Plasmids pmNEg1, 3, and 4 are the products of directional subcloning. The orientation of the mouse DNA in the pmNEg2 subclone was determined through restriction fragment analysis. The proposed gene targeting construct is based upon the pPNT vector (Fig 3.2C), in which the hygromycin B phosphotransferase gene (hyg) serves as a positive selectable marker and the herpes simplex virus thymidine kinase (HSV-TK) gene is used to enrich for clones which undergo homologous recombination.

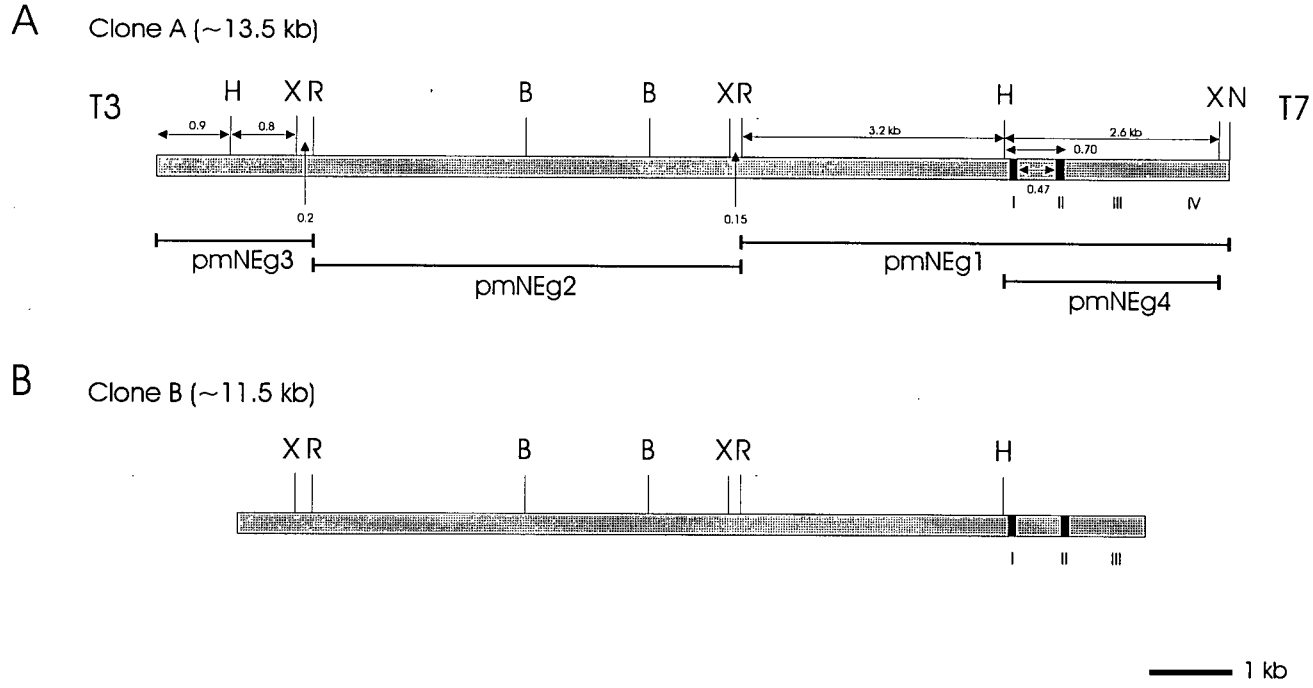


Figure 3.1- Physical map and organization of the mouse Ela2-positive Clones A and B. Restriction maps of clones A and B are derived from partial digestion/ indirect-end labelling restriction mapping together with direct restriction mapping of subcloned fragments pmNEg1 to 4 (thin horizontal lines). Restrictions sites are B,BamHI; H,HindIII; N, NotI; R,EcoRI; X,XbaI. The lightly shaded open boxes depict genomic mouse DNA. The filled boxes depict Ela2 exons Iand II; their locations and sizes (double and single arrows) weredetermined by PCR amplification of intervening and flanking non-coding sequences. The orientation of the clone relative to the lambda vector is indicated by the location of T3/T7 promoters of the Lambda Fix II arms. The size, in kilobase pairs, is indicated by a short bar. Refer to text for additional notes on cloning and characterization.

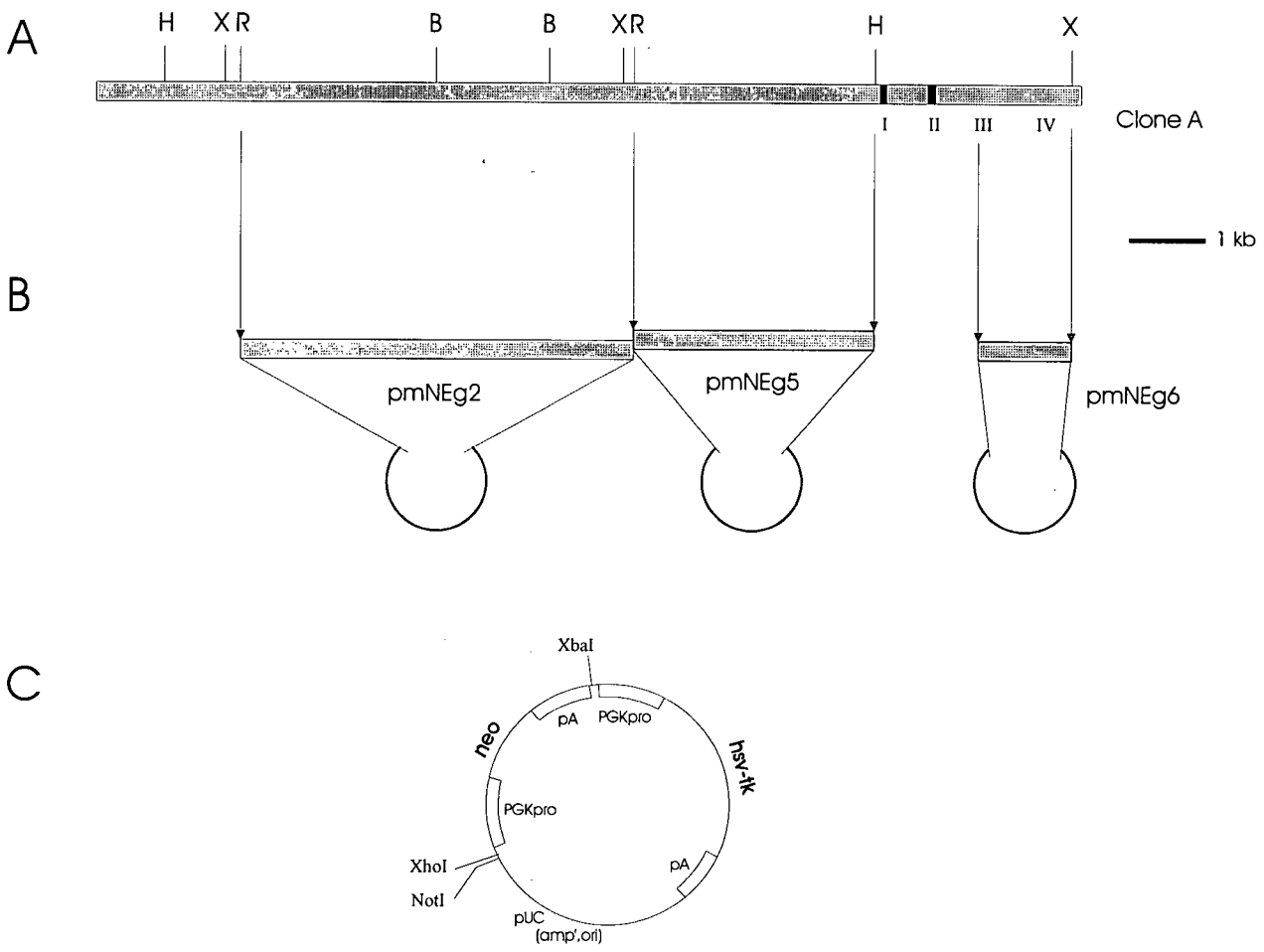


Figure 3.2- Schematic diagram of partial and proposed construction of the mouse NE gene targetting construct.
(A) Open boxes represent genomic DNA (to scale), and filled boxes illustrate location of the mouse NE exons I and II. Location of restriction sites used in construction of the plasmids in (B) are shown by short vertical lines (B,BamHI; H,HindIII; R,EcoRI; X,XbaI), with the exception of proposed pmNEg6, which was to be amplified from Clone A using a primer derived from mouse Ela2 exon III (MNE5) and a T7 primer (T7 site in Lambda arm). Subcloning to the 7.2 kb pPNT vector (C) is facilitated by directional subcloning of the pmNEg5 [EcoRI-HindIII] insert into pmNEg2, and the entire fragment is released and subcloned directionally into the XhoI/ NotI site of pPNT. The pmNEg6 would be subcloned into the XbaI site in the completed construct.

Results and Discussion

Cloning and characterization

Genomic mapping and isolation of the mouse NE gene is a prerequisite for the construction of a NE-deficient mouse. To clone mouse genomic NE, approximately 50 positive plaques were identified from two sets of ~1.5 x 10^6 plaque forming units (pfu) in a primary screening of a lambda genomic DNA library with radiolabeled mouse NE cDNA probe. Secondary and tertiary screens with the same probe revealed two true-positive clones: Agarose gel electrophoresis demonstrated that 'clone A' is ~13.5 kb and 'clone B' is ~11.5 kb. Restriction mapping with T3 and T7 oligonucleotides on BamHI, EcoRI, HindIII, and XbaI partial digests of Clones A and B demonstrated similar restriction site patterns between the two clones (Fig 3.1A/B), indicating that Clone B is a subset of Clone A. To determine the approximate location of the mouse NE gene in clones A and B, radiolabeled 5' and 3' fragments of the mouse NE cDNA (5' XbaI end to PstI site, first 152 bp; 3' XbaI end to RsaI site, last 200 bp) were used as probes to hybridize to Southern blots of Hind III digested pmNEg1, BamHI digested pmNEg2 and pmNEg3, and to XbaI, BamHI, EcoRI and HindIII digested Clone A. The 5' (exons I and II) but not 3' (exon V) mouse NE cDNA probes hybridized to a region of Clone A delimited by a HindIII restriction site and the T7 end of the clone, encompassed within the pmNEg1 subclone (Fig 3.1A), demonstrating that the 5' portion of the mouse NE gene is present in Clone A and furthermore is located at least in part within the HindIII/NotI fragment at the T7 end of the clone. This observation was confirmed by selective hybridization of the probe to the pmNEg4 subclone; hybridization to other regions of clone A was not observed, indicating that the T3 end of Clone A corresponds to the region 5' of the mouse NE gene locus. Lack of hybridization of the 3' end (exon V) probe provides preliminary evidence that the 3' end of mouse NE lies outside of Clone A. Reverse Southern hybridization of pmNEg1, 2, and 3 demonstrated hybridization of radiolabeled genomic probe DNA to pmNEg1 and 2, but not to pmNEg3 (not shown), indicating the presence of repetitive elements in pmNEg1 and 2.

To establish more precisely the location of the exons of the mouse NE gene, PCR amplification was used to determine the size and relative locations of the exons. PCR amplification of pmNEg4 with the MNE1 and MNE3 primer-pair (see Appendix, page 166), corresponding to sequences within mouse NE exons II and I, respectively, and alternately paired with T7 or T3 oligonucleotides (within the pBS polylinker region) revealed the putative location and orientation of exons I and II to the T7 and T3 sites of pmNEg4 (Fig 3.1A). A 0.70 kb fragment was amplified with the MNE1/T7 primer pair, revealing the position of exon II from the T7 site of the pBS polylinker, and adjacent to the Hind III site of Clone A. The MNE1/ MNE3 amplified intron I was 0.55 kb in length and was partially sequenced with the MNE1 primer, identifying the intron I/ exon II junction: [5'-...tcttctttgtgtgttctcag GCC CAG CAC TGG CCT CAG...-3']. The exon II sequence is identical to the reported mouse NE cDNA sequence, directly confirming the presence of the mouse NE gene in Clone A. Cloning of the complete mouse NE gene has been recently described (Belaaouaj et al. 1997); however, an extensive restriction site map was not provided by this group and thus a comparison with Clone A cannot be made. As expected, the exon/intron organization is similar to the human gene (Takahashi et al. 1988b). As the proteinase 3 and azurocidin genes exist together in a tightly linked cluster in humans (Jenne, 1994), a similar arrangement may also exist at the mouse locus. Data from low stringency hybridization of a human proteinase-3 cDNA probe to pmNEg1-4 suggested the possible presence of proteinase-3 in Clone A; however, the location of this gene was not confirmed.

Targeting construct

The initial construction of the targeting construct is outlined in Figure 3.2A/B. Only the 5' fragment of the mouse NE gene was targeted. Exon II contains a conserved histidine codon which is required for the proteolytic activity of human NE. Displacing exons I and II would produce a proteolytically inactive protein. Greater than 1 kb of Clone A (consisting of the 3' end of the gene) is thus available for use as a 3' flanking homologous region in the targeting vector. Additional features of the vector

construction strategy are detailed in the figure. As mentioned, successful targeting of the mouse Ela2 gene was recently reported (Belaaouaj et al. 1998). Further studies on these mice will undoubtedly provide important insight into the possible role of NE in the pathogenesis of emphysema in humans.

Summary of Part I

Although the etiological factors involved in the development of emphysema are clear, the pathogenesis of the disease is not. The association between α1AT-deficiency and emphysema suggests unbalanced proteolytic activity as a primary factor in development of the disease; however, whether this holds true for cigarette smoke-induced centrilobular emphysema is uncertain. In the absence of other convincing evidence, the observations associated with α1AT-deficiency have remained central to our understanding of smoking-induced emphysema. Oxidation and connective tissue repair have also been suggested to have potential implications in emphysema but have not been as extensively explored. A primary impediment in the elucidation of the pathogenesis of emphysema has been the lack of suitable relevant model systems to examine the potential of endogenously expressed proteases to produce emphysema. Transgenesis is a potentially useful technique that could overcome a number of shortcomings inherent to the commonly used intratracheal technique. The haptoglobin construction reported by D'Armiento suggested a useful approach but was demonstrated to be of limited applicability due to toxicity. Directing expression of NE to its endogenous compartment has also proven infeasible due to a lack of understanding of the mechanisms governing the transcription of this gene. A second issue is that the potential of a substance to induce emphysema does not imply a definite role for that substance in the development of emphysema. The recent advent of gene-targeting in mammals has presented a means of addressing this issue. The approach is tedious however, and this technique will perhaps be limited to the study of genes, such as NE, which already have shown substantial evidence of participation in the dis-

ease. Gene knock-out experiments would be most meaningful in comparison with other knock-outs (e.g. macrophage metalloproteinase vs. neutrophil elastase vs. cathepsin G, etc.). A more strategic approach would be to develop a more general knock-out model, for instance, in which the entire neutrophil lineage is absent or dysfunctional. The susceptibility of this animal to experimentally induced emphysema could be compared to an animal in which the alveolar macrophage lineage is absent and could thus demonstrate the relative contribution of macrophages versus neutrophils to the disease. Animals with defective chemoattractive or leukocyte adhesion/ extravasation mechanisms (e.g., P-, L-, and E-selectin deficient mice (Jung and Ley, 1999)) could potentially be used for this purpose. Similarly, various transcription factor or cytokine and chemokine knock-outs can be of use. PU.1 deficient mice for example, have a reduced myeloid cell population (DeKoter et al. 1998). An important consideration is that obvious and subtle defects in genetically altered animals may exist which could limit the usefulness of interpretations stemming from such experiments. Targeting of lineage specific cytotoxic "suicide" constructs is another means of ablating the neutrophil lineage, but would depend upon the identification of cis-regulatory elements directing highly restricted neutrophil-specific expression.

Part 2. Assessing the Possible Role of Chromatin Organization in Regulation of Expression of the Neutrophil Elastase Gene

Chapter 4: Introduction

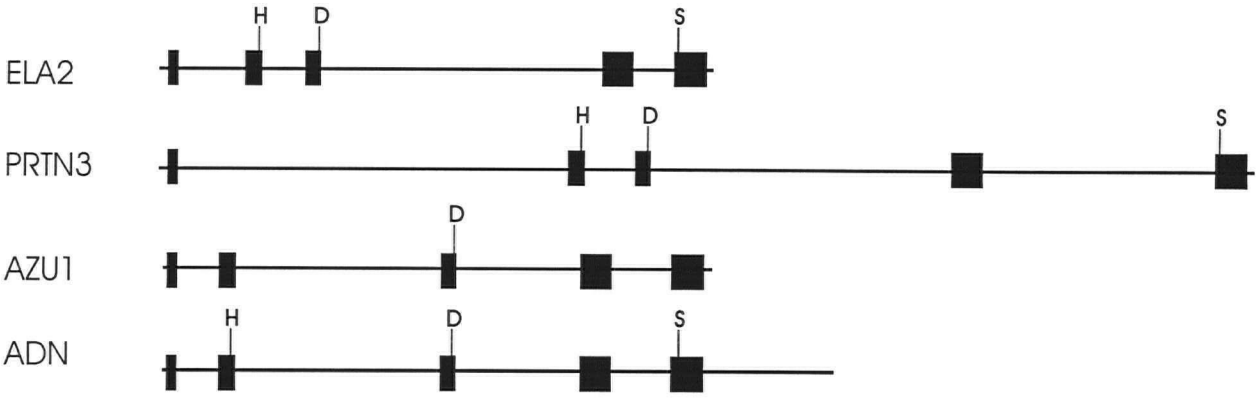
Although neutrophil elastase has been extensively studied as a pathogenic agent, the mechanisms regulating the expression of this protease have not been extensively studied. Part 2 of this thesis describes:

1. the current understanding regarding the regulation of neutrophil elastase expression and deficiencies in this understanding,
2. the potential role of chromatin structure in regulation of neutrophil elastase expression,
3. an assessment of the chromatin structure at the neutrophil elastase locus through identification of DNase I hypersensitive sites, and
4. a study of the association between chromatin structure and transcriptional regulatory activity of selected DNase I hypersensitive sites.

NEUTROPHIL ELASTASE GENE- STRUCTURE

Human neutrophil elastase (NE, also leukocyte elastase, polymorphonuclear leukocyte elastase, or medullasin) is a 29 kDa glycoprotein found in the azurophilic granules of neutrophils and is a major constituent (~ 3 pg per cell) of these granules (Takahashi et al. 1988b). The biological and pathological significance of NE has been discussed above (Part I). NE is classified structurally as a class 6 member of the trypsin superfamily of proteins, sharing similarities at the genomic and protein level with a number of immune effector cell proteases, including proteinase 3, adipsin, cathepsin G, mast cell chymase 1 and granzymes A, B, and H (Fig 4.1A and Jenne, 1994). The gene encoding NE has been cloned and sequenced in humans (gene symbol, ELA2; Takahashi et al. 1988b) and in mice (gene symbol, Ela2)

A



B

ELA2	MTLGRRLACLFLACVLPALLLGGTALA	SE	IVGGRRARPH
PRTN3	MAHRPPSPALASVLLALLLSAARA	AE	IVGGHEAQPH
AZU1	MTRLTVLALLAGLLASSRASSPL	LD	IVGGRKARPR
ADN	MHSSVYFAVLVLLGAAACAARPR	GR	ILGGREAEAH
CTSG	MQPLLLLLAFLLPTGAEA	GE	IIGGRESRPH
GZMA	MRNSYRFLASSLSVVVSLLLIPEDVC	EK	IIGGNEVTPH

Figure 4.1- Class 6 protease family gene structure.
(A) The exon (closed boxes)-intron (thin lines) organization between the human neutrophil elastase (ELA2), proteinase 3 (PRTN3), azurocidin (AZU1), and adipsin (ADN) genes are conserved and encode similar sized proteins (~25 kDa) The locations of the active site residues are indicated. Note that AZU1 lacks the His(57) and Ser(195) and therefore does not possess proteolytic activity. (B) Amino acid alignment of ELA2, PRTN3, AZU1, ADN, CTSG (cathepsin G), and GZMA (granzyme A) amino termini in unprocessed, precursor proteins. The shaded boxes indicate the 'pre'-signal peptide, followed by a 'pro' dipeptide.

(Farley et al. 1989; Nuchprayoon et al. 1994; Belaaouaj et al. 1997). Mouse and human NE share 71% similarity at the amino acid level (BLASTp). ELA2 is composed of 5 exons spanning approximately 4 kb, encoding a 267 amino acid pre-proelastase consisting of an N-terminal 27 amino acid signal peptide, a Ser-Glu dipeptide, and a 20 amino acid C-terminal extension, which are cleaved to form the mature protein (Fig 4.1B). The dipeptide structure is common to a number of protease zymogens and is also found in cathepsin G, granzymes, and the pancreatic serine proteases, allowing sorting and transport of these proteases in an inactive form, presumably to prevent proteolytic damage to protein synthesis and transport machinery. The dipeptide is cleaved in the azurophilic granule by dipeptidyl peptidase (cathepsin C) and the protease is stored in an active form in these granules. Granule targeting has been shown to be mediated by temporal expression patterns during granulopoiesis rather than sorting information encoded at the protein level (Lecabec et al. 1996; Arnljots et al. 1998). Targeting to azurophilic granules occurs at the promyelocyte stage whereas expression at the subsequent myelocyte stage results in targeting to specific granules. Expression at the incorrect time-point would thus cause the protease to be distributed to an incorrect compartment, emphasizing the importance of temporal control of gene regulation of the neutrophil granule proteins in addition to their highly restricted tissue-specific expression.

ELA2 has been localized to the telomeric region of the short arm of chromosome 19 in humans (19p13.3) (Fig 4.2A; Zimmer et al. 1992). In mice, Ela2 is found on chromosome 10 (Belaaouaj et al. 1997). The human gene, ELA2, is clustered in a region of 50 kb with the genes encoding azurocidin (also CAP37; human gene symbol, AZU1) and proteinase 3 (also Wegener's autoantigen, myeloblastin, p29b, or AGP7; human gene symbol, PRTN3) (Fig 4.2B), with which it shares 52% identity and 69% similarity, and 43% identity and 58% similarity, respectively (BLASTp). All three genes consist of 5 exons and possess additional similarities in primary structure (Fig 4.1A), suggesting that they were derived from a common predecessor (Jenne, 1994). Both proteinase 3 and azurocidin proteins were originally

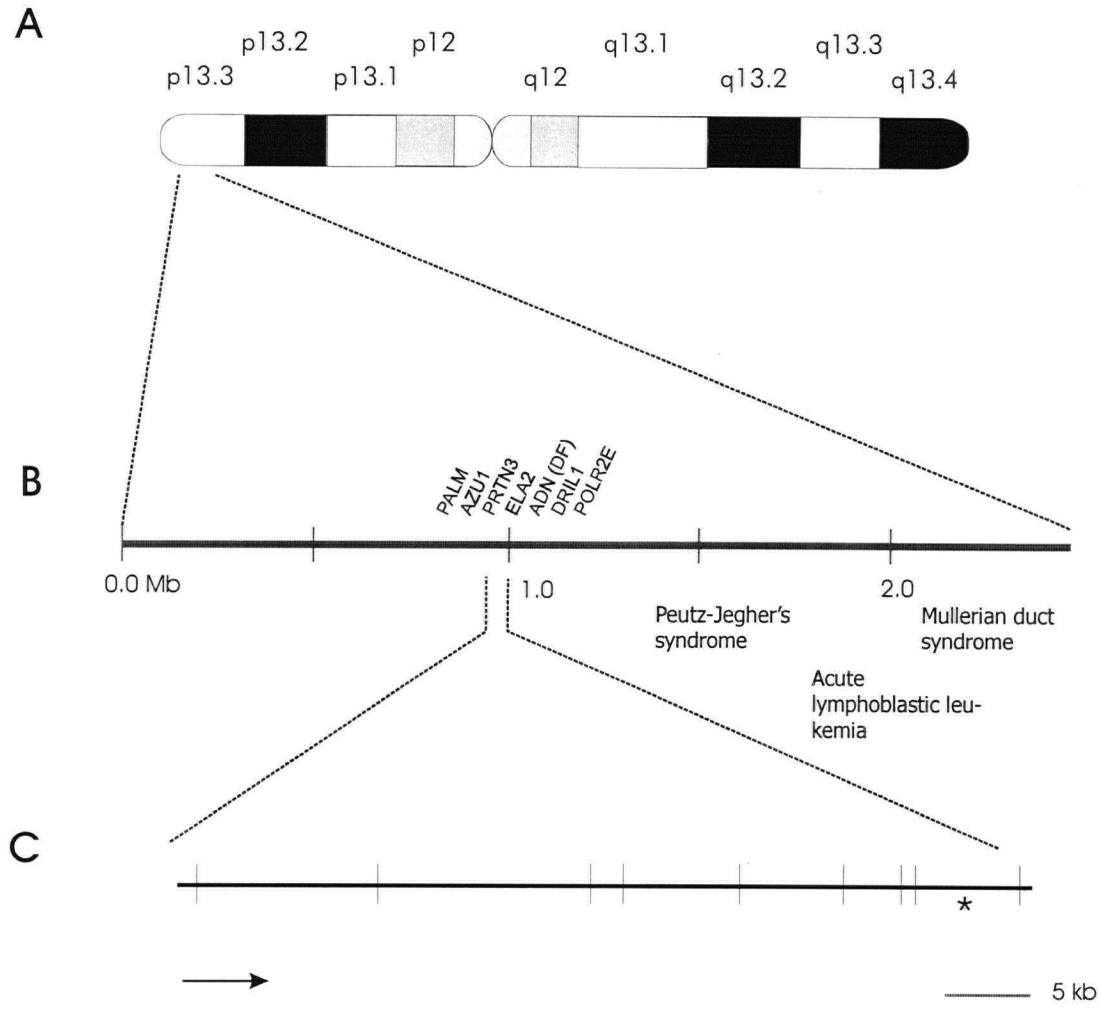


Figure 4.2- Schematic diagram of the APEA locus in the chromosome 19p13.3 region. (A) Cytogenetic and gene ideogram of human chromosome 19. (B) The 13.3 band is ≈8 Mb in length, and the azurocidin-proteinase 3-neutrophil elastase-adipsin (APEA) gene cluster lies approximately 1Mb from the p-telomere end. Gene symbols: POLR2E, RNA polymerase II polypeptide E; DRIL1, dead ringer (Drosophila)/ bright-like 1 homolog; ADN (DF), adipsin; AZU1, azurocidin; ELA2, neutrophil elastase; PALM, paralemmin; PRTN3, proteinase 3. Genes are in order indicated, but their exact location and relative distance from each other is not shown here. Known disease loci are indicated. (C) EcoRI restriction map of cosmid R33516, encompassing AZU1, PRTN3, and part of ELA2; thin vertical lines represent EcoRI restriction sites; asterix indicates ELA2-positive fragment; the arrow points in the transcriptional direction (5′ to 3′) of the AZU1, PRTN3, ELA2, and ADN genes; the entire cosmid is ~0.05 Mb. Data are preliminary observations of the Lawrence Livermore Human Genome Center chromosome 19 project obtained through chromosome 19 positive cosmid fluorescence in situ hybridization to prometaphase chromosomes and to sperm pronuclei and further mapped through EcoRI restriction mapping and genetic linkage studies.

identified from fractions of neutrophil primary granules with microbicidal activity and represent primary components of these fractions (Gabay et al. 1989), attesting to the view that they are key mediators of neutrophil proteolytic and antimicrobial activity. Functionally, proteinase 3 was of additional interest as it was independently characterized as the antigen recognized by anti-neutrophil cytoplasmic auto-antibodies (C-ANCA) associated with Wegener's disease, a systemic necrotizing granulomatous vasculitis (reviewed in Jenne et al. 1990). A previous study had demonstrated that proteinase 3 expression affects myeloid cell differentiation (Bories et al. 1989) and transfection of antisense PRTN3 oligonucleotides to an early myeloid cell line induces these cells to differentiate. Proteinase 3 has been shown to truncate Sp1 (Rao et al. 1998), a transcription factor expressed ubiquitously in proliferating cells (Dynan and Tjian, 1993). By altering the transcription factor profile in the promyelocyte and affecting expression of growth regulatory proteins, proteinase 3 provides a possible mechanism through which the protease can affect cellular differentiation. Azurocidin does not exhibit proteolytic activity but has been shown to possess microbicidal activity which acts by increasing permeability of the bacterial cell wall and through an inherent monocyte chemotactic activity (Pereira et al. 1990).

NEUTROPHIL ELASTASE GENE- GENOMIC ORGANIZATION

In addition to AZU1 and PRTN3, the presence of the gene encoding adipsin (also complement factor D; human gene symbol, ADN) in the ELA2 gene cluster was identified recently by D.Jenne and M.Zimmer (Max Plank Institute, Münich) in a collaborative project of ours (below and Wong et al. 1999). Judging from structural similarities between the gene encoding adipsin and the AZU1, PRTN3, and ELA2 genes, ADN was hypothesized to be located in proximity to the ELA2 gene cluster. ADN furthermore exhibits a similar gene organization with the genes at the ELA2 locus, including similarities between exon- intron arrangement and length of putative propeptide sequence (Fig 4.1B)

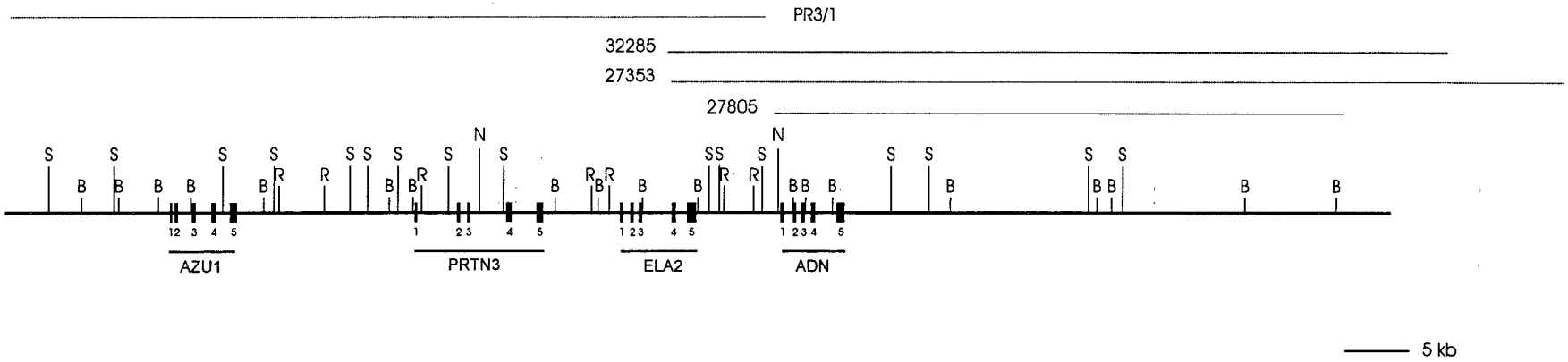


Figure 4.3- Physical scale map showing the organization of the AZU1-PRTN3-ELA2-ADN gene cluster.
The relative locations of the exons (closed boxes, designated by arabic numerals for each gene) are superimposed on the genomic region (thin horizontal line) as determined through restriction site analysis of the indicated cosmid clones (dotted lines, as indicated; refer to text). Restriction sites are depicted by short vertical lines (B,BamHI; N,NotI; R,EcoRI; S,SalI) as mapped by D.Jenne (Munich).

(Min and Spiegelman, 1986; Jenne, 1994). Screening of a chromosome 19-specific cosmid library with an adipsin-specific partial cDNA probe identified three cosmids, R27805, R32285 and R27353, which were shown to share identical restriction sites (Fig 4.3). Cosmid R32285 exhibited a restriction enzyme site pattern similar to a previously identified cosmid, PR3/1 at one end, which encompasses the ELA2 gene cluster (Zimmer et al. 1992) and was further characterized by restriction site mapping and Southern blotting using BamHI, EcoRI, Sal I, and Not I. Figure 4.3 shows the composite restriction site map for the three ADN-positive cosmids and PR3/1. The latter cosmid appears to overlap cosmid R32285 and R27353 by a few kb. To confirm this overlap, both ends of the R32285 insert were sequenced. One end was found to match the sequence of intron 3 of ELA2. Figure 4.3 also shows the location of the five ADN exons. Exon-intron junctions of the ADN gene and transcriptional orientation were similar to those of AZU1, PRTN3, and ELA2. ADN thus represents a fourth member of the chromosome 19p13.3 protease/ protease homologue gene cluster.

Unlike the other three genes at the locus, expression of the adipsin gene is not restricted to the myeloid lineage. Adipsin is present in the circulation at relatively high levels of 50 μg/ml (Cook et al. 1987; also Chapter 5). The enzyme is mainly synthesized and secreted by adipocytes and has been studied as a potential regulator of lipid metabolism. Monocytes and macrophages also express adipsin, which is already converted into the mature form before secretion (White et al. 1992). Adipsin/ factor D is a key regulator of the alternative pathway of the complement system with absolute substrate specificity for factor B as a component of the reversible C3bB complex. It only acquires its proteolytically active conformation during cleavage of the C3bB complex. Although adipsin has not been regarded as a genuine member of the hematopoietic serine protease gene family previously, exon-intron organization of ADN is completely conserved, indicating that ADN is derived from a common ancestor for hematopoietic serine proteases. In support of this view is the finding that adipsin is also expressed by monocytes and macrophages, but not by human or rodent hepatocytes, which synthesize all other components of the comple-

ment system. Furthermore, adipsin and the other neutrophil enzymes are still related in their biological functions in that all these serine protease homologs contribute to bactericidal defense mechanisms.

The work in Part II of this thesis focuses on this locus, which will subsequently be referred to as the 'NE/ ELA2 locus' or as the 'AZU1-PRTN3-ELA2-ADN (APEA) gene cluster'.

NEUTROPHIL ELASTASE GENE- GENETICS

Of the four proteins encoded by the APEA cluster of genes, only the mechanisms governing neutrophil elastase expression have been investigated in any significant detail. AZU1 and PRTN3 share a similar pattern of expression with ELA2 in that their expression also appears to be specific to the early stages of granulocyte myelopoiesis (Zimmer et al. 1992); however, it is unknown whether these genes possess similar transcriptional regulatory mechanisms. Expression of AZU1, PRTN3, and ELA2 is restricted to the myeloid lineage; expression is furthermore limited to bone marrow-derived blood cells and is not observed in leukocytes from peripheral blood (Takahashi et al. 1988a). In situ hybridization demonstrates that ELA2 expression is first apparent in myeloblast cells in marrow and subsides by the early myelocyte stage. ELA2 expression is most prominent at the promyelocyte stage however, and no transcripts can be detected in mature neutrophils (Fouret et al. 1989). AZU1 and PRTN3 share a similar pattern of expression with ELA2(Egesten et al. 1994). Athough monocytes are closely related to granulocytes in hematopoietic differentiation, being derived from an apparently shared myeloid precursor (possibly the CFU-GM), they do not carry neutrophil elastase.

Inspection of the 5' flanking sequence up to 1460 bp upstream (-1460) of the ELA2 transcription initiation site reveals 'TATA' and 'CAAT' box consensus sequences and a 53 bp element that is repeated

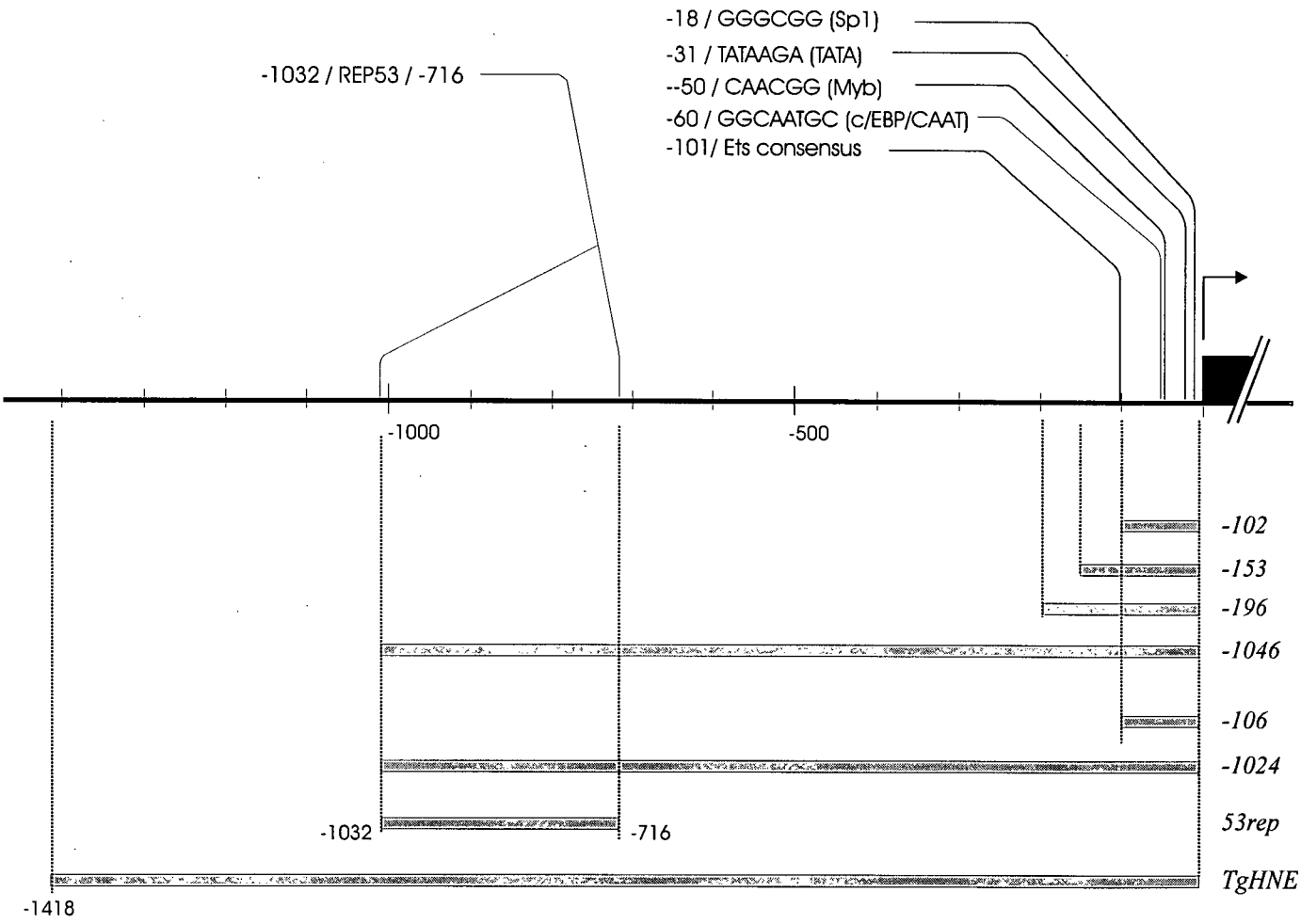


Figure 4.4- Regions of ELA2 5' flanking DNA previously examined for transcriptional promoter/ enhancer activity.

The location of transcription factor binding sites in the 5' flanking DNA (thin horizontal line) of ELA2 are shown. The regions previously examined are indicated (shaded boxes): Han et al. (1991) examined -102, -153, -196, and -1024; Srikanth and Rado (1994) examined -106 and -1024; Yoshimura et al. (1994) examined 53rep; TgHNE is examined in chapters 2 and 6. Transcriptional regulatory activity of these elements is described in the text.

7 times ('REP53', -1032 to -715) (Fig 4.4; Farley et al. 1989). There are also two regions (-130 to -112; -106 to -76) with 90% identity to homologous regions in the myeloperoxidase gene (-149 to -132 and +13880 to +13909, respectively) (Takahashi et al. 1988b). Conventional structure-function studies have been performed with ELA2. Transfection of a CAT reporter construct linked to deletion fragments of ELA2 5' flanking DNA between -1046 and the transcription initiation site into U-937 myeloid cells revealed a region (to -153) which substantially increased expression relative to a "promoterless" construct (Han et al. 1991) (see Fig 4.4). Deletion to -102 or addition of sequences to -196 or more (up to -1046), reduced reporter activity to background levels, suggesting that the 5' -153 bp region of ELA2 is a minimal promoter, and indicating the presence of a repressor element between -153 and -196. Several transcription factor binding motifs (C/EBP, c-Myb, and ets) are found in the -153 promoter element (Fig 4.4). The -153 promoter element was not functional in the non-myeloid (hepatoma) cell line HepG2, suggesting that the promoter confers myeloid-specific expression. Oddly, a subsequent report by the same group did not yield the same results and instead suggested that a -106 fragment was capable of conferring transcriptional activity to the CAT reporter vector and that inclusion of DNA up to -1024 (see Fig 4.4) had no additional effect on transcription, positive or negative (Srikanth and Rado, 1994). Similar results were also observed when these constructs were transfected into an erythro-myeloid (K562 erythroleukemia) cell line but not with HeLa cervical carcinoma cells, indicating a possible erythro-myeloid but not myeloid-restricted specificity. Since K-562 cells do not express neutrophil elastase (see Results , below), the cell-type specificity of this region does not correspond with the transcriptional activity of the gene. When linked to a chicken β-actin promoter, another group demonstrated that the 53 bp repetitive region (-1032 to -716, Fig 4.4) enhances transcription of a reporter gene when transiently transfected into either K-562 erythro-myeloid or HeLa cells (Yoshimura et al. 1994). The enhancer activity of the repetitive region in heterologous cell types contrasts with the conclusions from the other two studies, which did not detect enhancer activity of this region in myeloid cells.

More consistent results have been obtained by Friedman's group with murine Ela2. Transient transfection of Ela2 5' deletion constructs (to -1800) demonstrated a minimal region (to -91) which is needed to promote transcription of a luciferase reporter gene in G-CSF induced, NE-expressing (granulocyte differentiated, blast/ promyelocyte stage) murine 32D c13 cells, but not in non-induced, non-expressing cells (Nuchprayoon et al. 1994). Deletion to -79 reduced expression by 9 to 23-fold and deletion to -66 reduced expression further; clustered point mutations introduced into this region were used to corroborate these findings, demonstrating the presence of several transcription binding sites which are required for promoter function. A number of consensus transcription factor binding sites between mouse Ela2 -90 to -40 share significant homology with the similar region in human ELA2. These regions also share similar transcription factor binding sites. There is also sequence similarity between the 5' proximal regions of ELA2, AZU1 and PRTN3 (Chapter 6). Together, the functional studies with mouse Ela2 and human ELA2 indicate that a minimal, conserved, and possibly myeloid-specific promoter region exists within approximately 100 bp of the transcription initiation site, as determined by transient transfection assays.

CHROMATIN STRUCTURE AND GENE REGULATION

The observation that the 1.5 kb of DNA flanking the 5' end of ELA2 was unable to drive expression of the HNE transgene in mice (Chapter 2), whereas only -102 bp was needed to drive transcription in transient assays (Srikanth and Rado, 1994), is the first evidence of a potentially important role of chromatin structure in ELA2 expression. Whereas a transiently transfected construct is generally not incorporated into genomic DNA, transgenes are integrated and are therefore subject to the full effects of chromatin organization. In order to fully understand the mechanisms governing the expression of ELA2, it is necessary to elucidate how ELA2 is expressed in the presence of chromatin organization. The association between chromatin structure and gene expression is a well accepted phenomenon; however, the

mechanisms through which chromatin affects gene transcription is currently the subject of intensive investigation.

Chromatin structure and function

Chromatin structure is believed to directly affect gene expression, DNA replication, and other events involving DNA in eukaryotic nuclei (reviewed in Wolffe, 1992). Although a great deal is known about the structure of chromatin at the level of the nucleosome, chromatin structure is less well understood at higher levels of organization (reviewed in Grigoryev and Woodcock, 1998). Chromatin refers to the native state of genomic DNA, together with its associated proteins, in eukaryotic nuclei. The basic subunit of chromatin is the nucleosome, a nucleoprotein structure composed of ~200 bp of DNA (Richmond et al. 1984). The structure of the nucleosome has been resolved at 2.8 Å (Luger et al. 1997), confirming previous biochemical estimations that 146 bp of DNA is wound ~1.75 times around the histone octamer. The octamer consists of two molecules each of histones H2A, H2B, H3, and H4, which are highly conserved between eukaryotes. All histones possess a globular domain and a positively charged amino terminal tail which can be covalently modified by methylation, phosphorylation, or acetylation. The DNA between the core nucleosomes, linker DNA, is often associated with histone H1, which aids in the immobilization of the core histone octamer and is thought to facilitate the higher order folding of nucleosomal arrays (reviewed in Ramakrishnan, 1997). The nucleosome strand forms a 10 nm fiber which is in turn organized into higher-order structures that are less well defined (Felsenfeld, 1986); this organization of DNA results in an effective packing ratio of $\sim 10^3$ in interphase chromosomes and greater than 10^4 in metaphase chromosomes, suggesting a structural role for chromatin in the organization of DNA.

Chromatin and transcriptional activity.

Much progress has been made towards our understanding of the role of chromatin structure in gene expression over the past decade (the existence of the nucleosome, for example, was postulated only 25 years ago (Kornberg, 1974)), and a general understanding of the mechanisms whereby chromatin regulates gene expression is only beginning to be understood. The mechanisms of chromatin remodeling and its effect on gene expression are also beginning to be understood at the molecular level. The reader is referred to some of many general reviews on chromatin and its relationship to gene expression (Lewin, 1994a). A general overview of this area is provided here however, as a backdrop for experiments in Chapters 5 and 6.

Generally speaking, observations of chromatin structure and gene expression can be categorized into 1) mechanisms responsible for transcriptional silencing or 2) activation, and 3) mechanisms for separating the two. Different experimental approaches to understanding chromatin in gene expression, including studies of position-effect variegation, chromatin remodeling, heterochromatization/ silencing, insulators/ boundary elements, matrix-attachment regions/ nuclear localization, locus control regions, acetylation/ deacetylation, etc., can be placed in one of the three categories listed above and eventually, a unified understanding of how these different phenomena interact to mediate correct expression of a gene should be obtained. Chromatin organization is believed to serve important functional roles in gene expression. The extensive binding of DNA by histones into chromatin presents a prima facie view of chromatin organization as an impediment in the association of transcription factors and RNA polymerase with their cognate cis-elements in DNA and of mobility of RNA polymerase during transcription, intimating that chromatin is transcriptionally repressive. This view has been supported by numerous observations, both in vivo and in vitro (reviewed in Wolffe, 1992); the association between heterochromatin and transcriptional inactivity was an early supporting evidence of chromatin-mediated repression; a prominent example is the association between inactivation of the X-chromosome in mammals and chro-

matin condensation (Lyon, 1961). An emerging concept that is of particular interest to this thesis is that chromatin is not only a general repressor of transcription, but also plays an active role in mediating transcription at the level of individual cell types and loci. An initial observation supporting this view was the preferential sensitivity of the chicken globin gene in actively transcribing cells to digestion by a non-specific endonuclease (Weintraub and Groudine, 1976). Preferential general sensitivity to endonuclease digestion relative to the bulk of genomic DNA is considered to be indicative of destabilized or "open" chromatin structure, resulting in steric accessibility to DNA by the enzyme. Several additional loci have been analyzed in this manner with similar conclusions, including the chicken lysozyme locus (Jantzen et al. 1986), and the human β-globin gene cluster locus (Forrester et al. 1986). The region of open chromatin is generally observed to extend beyond the 5' and 3' ends of the gene and has been hypothesized to represent a *gene domain*, which can be defined as a region of DNA containing one or more complete genes and flanking DNA which together confers full structural and transcriptional integrity to that gene.

Nuclease hypersensitive sites.

Nuclease hypersensitive sites are an experimentally and conceptually important feature of chromatin. These are regions of DNA which are preferentially sensitive to hydrolysis by nucleases, relative to adjacent DNA. In contrast to regions of general nuclease sensitivity, which encompass the gene and extend continuously for several kb in the 5' and 3' directions, hypersensitive sites are short regions of approximately 200 bp that are superimposed upon areas of general sensitivity and, as their designation suggests, are distinctively more sensitive to nuclease digestion. The enzyme most commonly used for this purpose is the non-specific endonuclease deoxyribonuclease I (DNase I), and hypersensitive sites identified through the use of this enzyme are hence referred to as DNase I hypersensitive sites (DHS). Nuclease hypersensitive sites are believed to represent regions of DNA largely devoid of nucleosome formation. Initially identified as a feature of the eukaryotic genome in 1979 (Wu et al. 1979), DHS have since been

shown to be commonly associated with cis-active elements involved in transcription, replication, and recombination (Gross and Garrard, 1988).

Evidence for the molecular structure of DHS are derived from numerous observations, including an early observation that a DHS located approximately 260 bp 5' to the transcription initiation site of the chicken adult β-globin gene localizes to a region of DNA approximately 200 bp in length that is susceptible to cleavage by a number of endonucleases in addition to DNase I. In particular, cleavage with a restriction enzyme, MspI, releases a 115 bp fragment (McGhee et al. 1981) from this site, an observation that is inconsistent with the presence of a canonical nucleosome in this area. DHS thus appear to represent regions of DNA which are devoid or less tightly bound by histones in a regular nucleosome structure. Many nuclease hypersensitive sites are sensitive to S1 nuclease, suggesting that these regions may have single-stranded DNA properties (Larsen and Weintraub, 1982; Michelotti et al. 1996). Although nuclease hypersensitive sites may be devoid of nucleosomes in certain cases, only slight disruption of the regular nucleosomal structure appears to be sufficient to confer hypersensitivity. For instance, glucocorticoid receptor activation disrupts a regular nucleosomal arrangement at the mouse mammary tumor virus (MMTV) promoter with the induction of a DHS in which the DNA remains in contact with histones H2B (Bresnick et al. 1992).

DHS can be faithfully recreated in integrated transgenic DNA (Radice and Costantini, 1986), indicating that the information responsible for DHS-formation is encoded in DNA. Erythroid-specific DHS at the human β-globin locus (see below) which are absent in a human non-erythroid cell line can furthermore be induced by fusion of human somatic, non-erythroid cells with mouse erythroid cells. This also results in transcriptional activation of the inactive gene (Forrester et al. 1987). These observations indicate that trans-acting factors can be responsible for the induction of DHS. The loss of regular nucleosome organization at DHS can be caused by the binding of non-histone proteins such as transcription factors to the region, competitively inhibiting the binding of histones and thereby preventing formation of

a regular nucleosome. One of several studies specifically supporting this possibility demonstrated that formation of 5'HS4, a human β-globin upstream DHS depends on the presence of NF-E2 and GATA binding motifs within this site (Stamatoyannopoulos et al. 1995).

Chromatin remodeling

Three possible mechanisms have been proposed for the binding of transcription factors to chromatin-bound DNA (experimentally determined by sensitivity to nucleases such as DNaseI, micrococcal nuclease, S1 nuclease, or a specific restriction endonuclease):

1. The *dynamic competition* mechanism suggests that trans-acting factor binding to DNA can occur in the presence of chromatin through a passive mechanism, resulting in disruption of chromatin structure in the absence of DNA replication (e.g. Prioleau et al. 1994). Nucleosome positioning is believed to be important in facilitating the binding of transcription factors to their cognate elements in chromatin. Translational positioning refers to the location of a particular sequence of DNA as it enters the nucleosome. Differences in translational positioning would affect the DNA found in the linker region, which is believed to be more accessible to trans-acting factors (it is experimentally more susceptible to cleavage by micrococcal nuclease). Rotational positioning refers to rotation about the length of the DNA chain, and would preferentially expose any one of 10 bp at a particular turn of the helix. This could presumably be important in allowing the binding of transcription factors by exposing the transcription factor binding sequence. Translationally positioned nucleosome formation has been observed at several inducible promoters. The yeast acid phosphatase (PHO5) promoter is a well studied model where conserved nucleosome positioning is a demonstrated requirement for proper transcriptional activation (Schmid et al. 1992). Phosphate starvation activates a transcription and replication independent disruption of 4 positioned nucleosomes at the PHO5 promoter. One of 2 binding sites (UASp1) for the PHO4 transactivating protein is exposed in a linker region. Binding of PHO4 to UASp1 is a requirement for nucleosomal dis-

ruption, followed by binding to a second (UASp2) and third (Pho2) site, and transcriptional activation (Venter et al. 1994). The MMTV promoter offers another example of translationally positioned nucleosomes affecting transcriptional activity (Venditti et al. 1998). Although nucleosome formation and hence chromatin is often considered repressive of transcriptional activity, as discussed, phased nucleosomal arrays have been demonstrated to be important in facilitating gene transcription; for instance, a positioned nucleosome at the Drosophila Adh proximal 5' region juxtaposes a distal enhancer with the promoter region (Jackson and Benyajati, 1993).

2. The *pre-emptive* model suggests that replication is required for chromatin remodeling, and postulates that the displacement of histones from DNA during replication allows for competition between transcription factor and histone binding. Classical in vitro reconstitution studies support this view, as they show that sequential binding of transcription factors to a DNA template followed by reconstitution of nucleosomes allows transcription, whereas initial binding of histones precludes the binding of transcription factors and prevents transcriptional activation (summarized in Lewin, 1994b). In theory, DNA replication offers a window of opportunity in which histones are released from the DNA, presumably allowing this sort of competition.

3. A few examples of *active chromatin remodeling* (ATP-dependent) have recently been described, and such mechanisms are now hypothesized to be important for transcriptional activation in general. The most well-studied example of a chromatin remodeling machine is yeast SWI/SNF, a complex consisting of about 10 proteins. One component, the SWI2/SNF2 protein, possesses ATPase activity (Laurent et al. 1993). SWI/SNF is capable of remodeling a prearranged, in vitro nucleosomal array in an ATP-dependent fashion (Owen-Hughes et al. 1996), resulting in persistent disruption of the nucleosomal array as determined by DHS analysis*. The finding that TAF30, a component of TFIID, is also a SWI/SNF

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* N.B.: This suggests that active remodeling of nucleosomes by SWI/SNF-like factors could be a mechanism responsible for the formation of DHS in vivo.

component (Wilson et al. 1996), suggests a mechanism whereby TAF30 mediates preferential targeting of the SWI/SNF complex to the promoter complex in order to facilitate transcription. SWI/SNF complex and SWI2/SNF2 gene homologs have been identified in other organisms, including Drosophila and humans (Wang et al. 1996), suggesting an important role of SWI/SNF and related remodeling complexes in eukaryotic gene regulation.

Another intensively studied nucleosome remodeling machine was identified on the basis of its ability to disrupt a regular nucleosomal array at the *Drosophila* hsp70 promoter (Tsukiyama et al. 1994). The hsp70 promoter contains GAGA binding sites and addition of GAGA factor has been shown to mediate nucleosome disruption and formation of a DHS at -100 relative to the transcription initation site in an ATP-dependent fashion. This remodeling activity is conferred by NURF (nucleosome remodeling factor) (Tsukiyama and Wu, 1995b), a complex of 4 proteins in which ISWI (imitation switch) is the ATP-utilizing component (Tsukiyama et al. 1995a). ISWI had been cloned originally based upon homology to brahma, the SWI2/SNF2 homolog in higher eukaryotes and a transcriptional activator. The association of NURF with a transcriptional activator homolog suggests that NURF can potentiate enhancer activity by disrupting chromatin organization at an enhancer. Numerous other ATP-dependent chromatin remodeling protein complexes have been subsequently identified with homology of the ATP-utilizing component to either SWI2/SNF2 or ISWI (reviewed in Wu et al. 1998, Kornberg and Lorch, 1999, and Imbalzano, 1998).

The human β-globin locus

Although correlations between chromatin and gene expression have been established and the mechanisms underlying chromatin-mediated silencing and chromatin remodeling are beginning to be defined, the relationship between these specific observations and gene expression in general are not well understood in most cases. The relationship between chromatin structure and its effect on transcription at the β-globin

gene locus is briefly discussed here since it is one of first eukaryotic gene loci that has been intensively studied from the perspective of chromatin structure in gene regulation and remains one of the most extensively studied gene loci in this field. Much of our understanding of the role of chromatin organization in the *in vivo* expression at the level of an entire gene locus has been derived from, or is represented by this model. Adding to the significance of this discussion is the fact that the β-globin locus shares similarities with the ELA2 gene locus:

1. The β-globin gene, like the ELA2 gene, exists in a cluster of structurally and evolutionarily related genes.
2. Expression of the genes in the β-globin locus is hematopoietic-specific.
3. Comparable model systems exist (cell lines arrested at an early stage of differentiation) which can be used to examine the expression of these genes.

There are also notable differences between the two loci: β-globin genes are expressed in erythroid but not myeloid cells, expression of the β-globin genes is development-specific rather than coordinated (below), and naturally-occurring mutations of the β-globin locus exist which have helped to define relevant regions of transcriptional regulatory activity, but no natural mutations have been reported at the ELA2 locus.

The β-globin gene cluster consists of five globin genes: 5'-ε-γ^G-γ^A-δ-β-3' that are arranged in their chronological order of expression during hematopoietic development from the embryo to adult (Collins and Weisman, 1984). These genes span ~55 kb, and lie within an extended region of general DNase I sensitivity (Forrester et al. 1986). A series of DHS are observed at this locus; importantly, 5 particularly strong sites exist at -21(5), -17.5(4), -14.5(3), -11(2), and -6(1) kb from the ε-globin gene (Tuan et al. 1985; Forrester et al. 1986). HS 5 is constitutive, while HS 4, 3, 2, and 1 are erythroid specific.

One of the first observations that the 5' β-globin DHS are important in regulating β-globin gene expression was the finding that Hispanic γδβ-thallasemia, in which the γ, δ, and β globin genes are not expressed, results from a deletion from -9.5 to -39 kb upstream of the 5'-most (epsilon) gene (Driscoll et al. 1989). The deletion in this area results in loss of DNase I general sensitivity at the β-globin locus (Forrester et al. 1990), indicating an important role of this region in generating an open chromatin conformation. YAC-transfer of a 150 kb region encompassing the β-globin gene cluster recapitulates correct endogenous expression (Porcu et al. 1997), indicating that the information necessary for directing correct endogenous expression of the cluster of genes resides within this area. Finer functional mapping in transgenic mice indicate that HS 1 to 4 coordinately confer levels of expression similar to endogenous levels of gene expression in a copy-number correlated, transgene integration site-independent fashion (Forrester et al. 1987); regions with such activity are referred to as LCRs (locus control regions). Mutations of any of the HS-1 to 5 elements in a YAC β-globin transgene also have been demonstrated to reduce the ability of the LCR to confer an open chromatin conformation at the locus (Li et al. 1998). Another study did not confirm this (Reik et al. 1998).

HS2 exhibits LCR activity in some studies (Philipsen et al. 1990; Talbot et al. 1990), but not in others (Ellis et al. 1996; Caterina et al. 1994); however, deletion of HS 2 does not greatly affect developmental expression of the genes in the cluster (Peterson et al. 1996). HS 3 has dominant chromatin opening activity (Ellis et al. 1996) and is the most active of HS within the LCR during the embryonic period (Fraser et al. 1993). One study suggested that position-dependence is an important feature of HS3 (Philipsen et al. 1993) while another study maintains that position independence is not a feature of HS3 (Navas et al. 1995). Deletion of HS3 causes loss of correct ε and γ globin temporal expression during development and loss of position independence of expression (Navas et al. 1998), supporting a role for HS3 in up-regulating embryonic and fetal globin expression (Bungert et al. 1995). HS 4 deletion disrupts proper developmental regulation of the globin genes, but can be replaced by HS 3 (Bungert et al.

1995). It shows greatest activity in adult expression (Fraser et al. 1993). HS 5 is a chromatin insulator with little enhancer activity (Li and Stamatoyannopoulos, 1994) and likely serves as a boundary element. Competition with other promoters at the locus (Anderson et al. 1993; Kim et al. 1992) occurs, suggesting a model where globin gene switching during development is mediated by competition for interaction with the LCR. Abundance of transcription factors and distance from the LCR also affect which gene is transcribed (Dillon et al. 1997).

Several features inherent to the LCR of the β-globin gene domain are thought to be essential to eukaryotic gene loci in general. These include boundary elements which delimit the gene locus, preventing the influences of neighboring chromatin and *vice versa*; an element capable of potentiating the domain by opening the chromatin at the locus, and of course, the conventional proximal promoter and enhancer elements which are in part responsible for tissue-specificity.

Silencing

As discussed, chromatin is generally repressive of transcription and mechanisms must exist to allow for correct transcriptional activation during development and differentiation in the correct cell type/s. Conversely, genes must be repressed in certain tissues and at certain stages in development, during differentiation, or in response to environmental stimuli. Chromatin-mediated repression is an obvious mechanism of transcriptional silencing in eukaryotes, preventing aberrant gene transcription. The formation of heterochromatin at telomeres and centromeres is constitutive and is presumably due to the presence of simple repetitive sequences (see Wolffe, 1992); however, reversible silencing at gene loci is less well understood. The most well characterized example of locus-specific silencing is that of the silent yeast mating type loci HML and HMR (reviewed in Lewin, 1994b). This locus is constitutively inactive, and silencing elements at these loci are necessary and sufficient to direct silencing in this region (Brand et al. 1985). The phenomenon of position-effect variegation (PEV) is also presumed to be a manifestation of

silencing. PEV can be referred to as the epigenetic, clonally-inherited somatic transcriptional state of certain genes, depending upon their site of integration. Integration of genes near a telomere often results in PEV, with clonal activation or silencing of the integrated gene. A prominent example is the X-chromosome inversion of the *white* locus (w^+) in Drosophila, which results in juxtaposition of the locus to within 25 kb of heterochromatin, giving rise to PEV of *white* expression in the eye (reviewed in Weiler and Wakimoto, 1995). Some of the mechanisms regulating transcriptional silencing have been characterized recently at the molecular level. A large number of mutations have been identified which affect PEV by increasing or decreasing variegation (see Weiler and Wakimoto, 1995). Most of the PEV modifying mutations affect chromosomal proteins or proteins which affect chromosomal proteins (Reuter and Spierer, 1992), including HP1, a pericentric heterochromatin-associated protein (Eissenberg et al. 1990). The SIR (silent information regulator) family of proteins are also involved in silencing of the mating type loci. Mutation of the SIR genes results in depression of these genes (Aparicio et al. 1991). Overexpression of SIR however, expands the silenced region of telomeres (Hecht et al. 1996), and this effect can extend for over 10 kb (Renauld et al. 1993), indicating that SIR proteins are involved in mediating the spread of transcriptional silencing. Telomeric Rap1 protein binding sites are found in yeast silent mating type loci (and at telomeres), and recruit SIR2, 3, and 4 (Marcand et al. 1996), indicating that mediators of silencing can be recruited to specific loci. SIR3 and SIR4 are believed to mediate silencing through interactions with the histone H3/ H4 tails (Hecht et al. 1995). Mutation of histones H3 and H4 have been shown to relieve silencing of HML and HMR (Thompson et al. 1994), further suggesting the importance of histone H3/ H4 in transcriptional silencing. A final note is that absence of transcriptional activation in itself could be an indirect mechanism of silencing in inducible or developmentally/ differentiation-specific gene regulation; this possibility suggests that the locus is in a transcriptionally repressed state by default. A combination of repression by default chromatin-formation and active heterochromatinization may be responsible for the proper transcriptional control of different genes. Histone

acetylation has received much recent attention as a means of transcriptional activation and deacetylation is thus a potential repressive mechanism. Several recent reviews describe associations between histone acetylation and the basal transcription machinery (e.g. $TAF_{II}250$), with repressors of transcription (e.g. mSin3A/B), and with chromatin assembly factors (e.g. CAF1) (refer to Grunstein, 1997), emphasizing an important role of acetylation and deacetylation in transcriptional regulation. Of particular interest in the area of silencing is the recent finding that histone deacetylase activity is associated with a methylated DNA-binding protein, MeCP2 (Nan et al., 1998), suggesting a mechanism whereby methylation induces silencing through a deacetylation of nucleosomes.

Insulators

As discussed, domain boundaries are postulated to exist at gene loci to distinguish the transcriptional state of chromatin within a gene domain from that residing outside of the domain. The observation that PEV can occur with translocated genes emphasizes the importance of elements which can insulate against the spread of inactive chromatin. Gene domains have been associated with three elements: matrix-attachment regions (MARs), LCRs, and insulators; these are not mutually exclusive (discussed further below).

Functional domain boundary elements were first demonstrated to exist at the Drosophila hsp70 locus, which is bordered by scs (specialized chromatin structure) and scs' elements; these elements are associated with distinct hypersensitive sites (Udvardy and Schedl, 1985). scs and scs' have been demonstrated to possess insulatory activity, capable of protecting a gene from position effects (Kellum and Schedl, 1991). An insulator can thus be defined experimentally by its ability to protect against position-effects of transgene integration and the ability to disrupt enhancer-promoter interactions when the insulator is placed between them. Insulatory activity is presumably a requirement of gene domain boundary elements. scs' is bound by BEAF-32A (boundary element associated factor) (Zhao et al. 1995), and

consensus repeats for BEAF-32A have been demonstrated to recreate insulatory activity. BEAF-32A itself is also found to localize to multiple sites in Drosophila polytene chromosomes, typically on one end of a puffed (transcriptionally active) region, suggesting a ubiquitous role for BEAF-32A in defining domain boundaries (Cuvier et al. 1998).

Another well-studied insulator is associated with the Drosophila *gypsy* retrotransposon. Insertions of the retrotransposon into the *yellow* (y) locus revealed the presence of insulatory activity at one end of the retrotransposon, and the elements responsible for this activity have been localized to a short region of DNA (Geyer et al. 1988). The su(Hw) protein binds to this sequence, and mutations of the binding sequence have been shown to abolish insulator activity (Holdridge and Dorsett, 1991). An associated protein, *mod(mdg4)* is essential for the insulatory activity of su(Hw) (Gerasimova et al. 1995). Mutations of *mod(mdg4)* increase insulatory activity, turning it into a repressive/ silencing element. This association between insulatory and silencing activity suggests that at least some insulators may function through a silencing-type mechanism (reviewed in Gerasimova and Corces, 1998).

Several hypotheses have been proposed, describing the potential mechanism of insulatory element activity:

1. The MAR hypothesis presents the possibility that nuclear matrix or scaffold attachment regions result in topologically constrained loops of chromatin, thus defining discrete physical gene domains (Phi-Van and Strätling, 1988).
2. The tracking model suggests that insulators form a physical boundary, preventing DNA binding protein/ enhancer complexes from scanning beyond it to interact with its promoter.
3. The transcriptional decoy model suggests that insulators disrupt enhancer-promoter interactions by binding the enhancer complex, competing against potential interaction with the promoter.

Summary

While conventional assays of proximal promoter regions of ELA2 have been well defined in transient assays, the important potential role of chromatin has not been addressed. Our initial studies with HNE in transgenic mice demonstrate repression of ELA2 expression and suggest that other elements are involved in creating a permissive environment for transcription. Studies suggest that at least several key elements are involved in defining correct gene expression in the presence of chromatin. Specific mechanisms of chromatin remodeling are being defined; however, the gap in our understanding between these basic mechanisms and the resulting tissue stage-specific expression of genes in mammals is an important area in which much remains to be understood.

Chapter 5- Chromatin Structure at the Neutrophil Elastase Locus in Myeloid Cells*

RATIONALE AND HYPOTHESIS

Functional elements at the ELA2 (human) and Ela2 (murine) 5' proximal flanking regions have been identified using transient transfection studies in cultured cells, as discussed, suggesting that this region mediates expression of these genes in myeloid cells. The interpretation of these findings is limited however, to the context of the experimental systems used. For instance, in contrast to these findings, we discovered from the experiments described in Chapter 2 that a 5' extended flanking region of ELA2 (up to 1.5 kb) is not sufficient to drive detectable levels of expression of the HNE transgene in 5 of 5 lines of transgenic mice, even when integrated in multiple copy numbers. The discrepancy between the previously described transient transfection studies and the transgenic data suggested that additional, unidentified features of the ELA2 gene are responsible for permitting the high endogenous levels of NE expression that are normally observed in human and mouse neutrophil precursors.

In contrast to transcriptional activity demonstrated in transient transfection systems, expression of a gene in vivo is complicated by effects of chromatin organization. A number of interrelated processes affect the endogenous expression of any eukaryotic gene, and these events can be broadly categorized as

1. assembly and mobilization of basal transcriptional machinery,
2. trans-activating factors and cis-acting elements
3. chromatin structure and modifications of chromatin (e.g. acetylation)

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* Data presented herein form the basis for a manuscript, Changes in chromatin organization at the neutrophil elastase locus associated with myeloid cell differentiation, by **Edmond T. L. Wong**, Dieter E. Jenne, Michael Zimmer, Susan D. Porter, and C. Blake Gilks. *Blood* (in press), 1999.

4. other epigenetic events such as DNA methylation

These processes are inter-related at different levels to define the correct spatial and temporal expression of a gene. Importantly, transient transfection experiments do not account for the very important effects of chromatin structure on transcription. Thus far, only the proximal promoter elements of ELA2 and Ela2 and their associated trans-acting factors have been studied (Chapter 4) and knowledge of the potential effects of chromatin on transcription of this gene was lacking until our studies with the HNE transgenic mice. The purpose of the work presented in this chapter is to provide some insight into the chromatin structure at the APEA locus. The underlying logic is that chromatin structure plays an integral role in transcription of eukaryotic genes.

Hypothesis:

Elucidation of the chromatin organization at the ELA2 locus through the identification of DNase I hypersensitive sites (DHS) will demonstrate regions of transcriptional regulatory activity involved in mediating correct spatial and temporal expression of the gene.

The work described in Chapter 5 is largely descriptive in nature and forms the basis for the work presented in Chapter 6. The specific experimental aims are as follows:

Specific Aim #1:

Determine the chromatin structure at the ELA2 locus in cells which actively transcribe the ELA2 gene (undifferentiated myeloid cells).

Specific Aim #2:

Assess possible differences in chromatin organization at the ELA2 locus between ELA2 expressing and non-expressing cells.

By describing the organization of chromatin at the ELA2 locus, a preliminary understanding of the effect of chromatin organization on transcriptional activity may be developed and could thus provide grounds for further work in elucidating the mechanisms governing high-level, promyelocyte-restricted expression of ELA2 in vivo (Chapter 6). From a more practical perspective, the myeloid compartment is particularly amenable to ex vivo manipulation (Emerson, 1996). In consideration of the abundance of neutrophils in the blood and their association with sites of inflammation, an improved understanding of the mechanisms governing myeloid-specific expression could eventually be of use in the development of gene therapy of this compartment for treatment of blood and particularly inflammatory disorders.

The approach taken here to understand the chromatin structure at the ELA2 locus is to map sites of nuclease hypersensitivity in this region. As described in Chapter 4, nuclease hypersensitive sites are regions of DNA with altered chromatin structure and are often associated with functional elements. By comparing the profile of DNase I hypersensitivity in U-937 cells to those of non-expressing cell types, an idea of the relationship between chromatin organization and transcriptional activity at this locus can be determined. DHS specific to expressing cells could then be further characterized (described further in Chapter 6). Although we are primarily concerned with the NE gene, the existence of ELA2 in a gene cluster suggests the possibility that the transcriptional regulation of the genes within the APEA locus may share common transcriptional regulatory elements. This supposition is further supported by the similarities in pattern of expression between AZU1, PRTN3, and ELA2 described above. In contrast, ADN lies within 3.5 kb on the 3' end of ELA2 but exhibits a pattern of expression distinct from the other genes at the locus. The mechanism allowing for coordinated regulation of AZU, PRTN3, ELA2 at one end of the locus and a distinct expression pattern at the other end remains to be understood. Thus, in studying the regulation of ELA2, it was considered prudent to examine the entire locus as the transcriptional regulation of this gene is likely to be influenced, or perhaps even shared, with the other genes at this locus.

METHODS

Cells

U-937 (ATCC CRL1593), HUT-78 (ATCC TIB161), Jurkat (ATCC TIB152), and K-562 (ATCC CCL243) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (fcs, Gibco BRL) and maintained at 37°C and 5% CO_2; HL-60 (ATCC CCL240) cells were cultured in IMDMEM with 20% fcs under the same conditions; COLO 201 cells (ATCC CCL224) were grown in RPMI 1640 medium supplemented with 20% fcs and maintained under the same conditions. For induction of differentiation, U-937 cells were cultured for 72 to 120 hours in RPMI 1640 supplemented with 10% fcs in the presence of 25 ng/ml TPA (12-o-tetradecanoyl-phorbol-13-acetate, also phorbol 12-myristate 13-acetate) (Gibco BRL). Polymorphonuclear granulocytes were isolated from heparinized donor blood from a healthy donor using Histopaque-1119 and 1077 (Sigma Diagnostics) double density gradient centrifugation and collection of the granulocyte population at the Histopaque 1119/1077 interface. The isolated cells were stained with H & E and examined under a light microscope to confirm the granulocyte population.

DNase I hypersensitive site mapping

For each cell line examined, approximately 10^7 cells from sub-confluent cultures of non-adherent cells were harvested by centrifugation at 500 x g for 5 min and washed twice in 1x PBS. Adherent cells were washed with 1x PBS, trypsinized, and washed with 1x PBS. The cell pellet was resuspended in 10 ml cold 1x RSB buffer (10mM Tris-Cl pH 7.5, 10mM NaCl, 3mM $MgCl_2$) with 0.50 to 0.75 %(v/v) NP-40 and homogenized with a 15 ml Dounce tissue grinder with a minimal pestle clearance of 70μm to disrupt the outer cell membrane. The homogenate was examined under a phase-contrast light microscope to determine completeness of outer cell membrane disruption and the homogenate was centrifuged at 700 x

g for 10 min to pellet the cell nuclei which were subsequently resuspended in cold 5 ml 1x RSB buffer and Dounce homogenized in a 7 ml tissue grinder with a minimal clearance of 50μm and stored on ice. For each DNase I time-point, 0.5 ml aliquots of the isolated nuclei were subjected to incremental DNase I digestion (depending on experimental determination of optimal range for each cell line examined; concentrations ranged from 0 to 50 U/ml (Boehringer Mannheim; approximately 5 μg/ml) for 10 min at 37°C, and the reactions were terminated with 0.5 ml stop buffer (1%(w/v) SDS, 0.6M NaCl, 20mM Tris-Cl pH7.5, 10mM EDTA, 0.4mg/ml proteinase K) and incubating at room temperature overnight. The DNase-treated nuclei were then extracted with phenol, 1:1 phenol-chloroform, and chloroform, precipitated with ethanol, and dissolved into 1x TE buffer for restriction enzyme digestion and analysis on Southern blot as indicated below.

Southern blotting

Restriction digested genomic DNA was separated by electrophoresis in 0.65 to 0.85 % TBE agarose, blotted onto Nytran Plus membrane (Schleicher and Schuell), UV light cross-linked, and hybridized with [α-^{32}P]dCTP random primed labeled DNA probes or with [α-^{32}P]UTP or CTP labeled riboprobes: Initial attempts to utilize genomic DNA as probes revealed the presence of numerous repetitive elements at the locus. Numerous fragments from cosmid Azi-13 (cosmid overlapping PR3/1; Fig 4.3) were subcloned and reverse Southern hybridization revealed few regions free of repetitive elements suitable for use as probe DNA within the locus, demonstrating the presence of abundant repetitive elements at the locus and reducing the value of non-coding DNA for use as unique probes. Recently posted sequencing data* (reviewed in retrospect) from the Lawrence Livermore genomic sequencing centre confirmed the presence of extensive repetitive elements dispersed throughout the intergenic and intronic regions in the

* LLNL Human Genome Center; sequencing clone R33516

regions examined and in other regions. The probes used were thus derived from AZU1, PRTN3, and ADN human cDNA clones (Zimmer et al. 1992), from the human ELA2 cDNA clone, a gift from M.Naruto (Kamakura, Japan) (Okano et al. 1990), and through standard PCR amplification or isolation by restriction digestion of sub-fragments within these cDNA clones as represented in figure 5.2. ELA2 exon I was PCR-amplified with BGR1/ BGR5 primer pair, ELA2 exons IV/V were isolated from ELA2 cDNA with PvuII restriction digestion and purification from agarose-gel electrophoresis-separated DNA, AZU1 exons IV/V and whole cDNA were obtained from Dr. Jenne (Munich), PRTN3 exon 4 was amplified with the PR3EX4A/B primer pair, and AZU1 exons 1-3 were isolated from a Nae I digestion of AZU cDNA. Hybridized blots were washed at a final stringency of 0.1 x SSC/ 0.1% SDS at 65°C for 2 hours and exposed to Kodak X-AR film at -70°C. The probes hybridized to single restriction fragments of the expected size when used to probe Southern blots under these conditions, confirming absence of cross-hybridization.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from cells with Trizol Reagent (Gibco BRL) according to the manufacturer's directions and examined by agarose gel electrophoresis to determine the integrity of the ribosomal bands before use. For Northern blot analysis, 10 μg of total RNA was denatured, separated on a 1% agarose-formaldehyde gel, transferred to Nytran Plus membrane, UV cross-linked and hybridized and washed as described above for Southern blots. The same blots were then stripped and hybridized with a human β-actin cDNA radiolabeled probe as described.

RESULTS AND DISCUSSION

To develop an understanding of the effect of chromatin organization on transcriptional activity at the ELA2 locus, we have examined the chromatin structure at this locus in cell lines representative of myeloid and non-myeloid cells. Here, we provide the first report of the characterization of chromatin structure at the APEA locus and determine a correlation between expression of the gene and the organization of chromatin at the locus.

Models for analysis of chromatin structure at the AZU1/ PRTN3/ ELA2/ ADN (APEA) locus.

Previous studies had demonstrated that NE is expressed in the promyelocyte population in human marrow and is also expressed in the U-937 cell line (Fouret et al. 1989; Takahashi et al. 1988a). U-937 cells are derived from a histiocytic lymphoma and exhibit morphological, biochemical, and antigenic features characteristic of early myeloid cells, indicating that they are arrested at an early stage of myeloid differentiation (Sundström and Nilsson, 1976). U-937 cells possibly correspond to the CFU-GM or late blast stage, although direct correlations cannot always be drawn for such malignant cell lines as isolation and establishment of a cell line is dependent upon its abnormal growth and differentiation characteristics. Due to practical limitations in isolating and culturing human hematopoietic stem cells (Spangrude, 1994), U-937 or a similar cell line, HL-60 (Collins et al. 1977), are commonly employed to study the genetic and biochemical events accompanying early myeloid cell development. U-937 cells were therefore used here to determine whether chromatin structure at the APEA locus is specific to cell lines which actively transcribe ELA2 and these findings were confirmed in HL-60 cells. Non-myeloid hematopoietic cell lines and a representative non-hematopoietic cell line were also examined: HUT-78 is a mature T cell line and Jurkat cells are lymphoid cells derived from an acute T cell leukemia. K-562 cells are derived from erythromyeloblastic cells arrested at an early stage of differentiation. COLO 201 cells are a non-hematopoietic, colonic epithelial cell line.

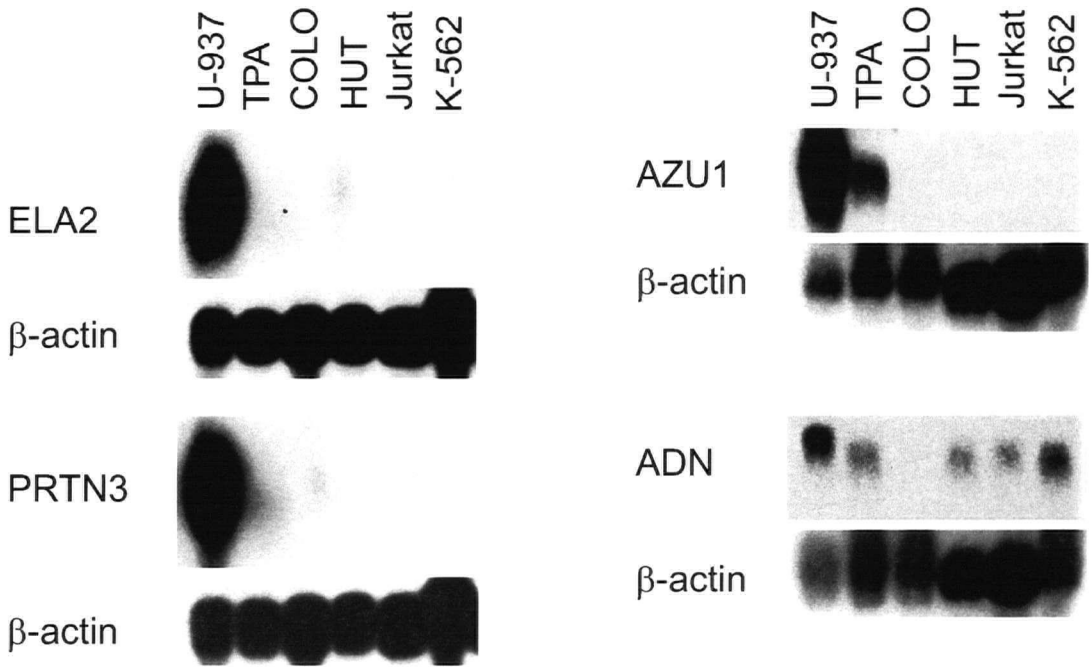

Figure 5.1- Expression of the AZU1-PRTN3-ELA2-ADN cluster of genes in cell lines studied.

Northern analysis was performed on U-937 cells, U-937 cells treated with 25 ng/mL TPA for 3 days (TPA), COLO 201 (COLO), HUT 78 (HUT), Jurkat, and K-562 cell lines and hybridized to ^{32}P-labeled human ELA2, AZU1, PRTN3, ADN, and human β-actin cDNA probes as indicated for each blot.

To determine that expression of NE is limited to myeloid cells in our model system, Northern analyses were performed. Among these cell lines, ELA2 transcription is restricted to U-937 cells (Fig 5.1). Treatment of U-937 cells with the phorbol ester TPA has been demonstrated to result in differentiation of these cells (Stockbauer et al. 1983), and transcription of AZU1, PRTN3, and ELA2 was shown to be coordinately decreased in TPA differentiated U-937 cells (Fig 5.1), as expected. While TPA-mediated down-regulation of ELA2 and PRTN3 in U-937 is complete in 3 days, I observed that the same cells still produce detectable though greatly reduced levels of AZU1 transcripts. A previous study did not detect differences in the levels of down-regulation between the three genes (Zimmer et al. 1992), and this discrepancy may be due to differences in sensitivity of the Northern analyses, differences between responses of U-937 sublines to prolonged TPA exposure, or to differences in culture conditions. As anticipated, ADN exhibits a less restricted pattern of expression than ELA2, PRTN3, and AZU1; however, ADN transcripts are found to be present in all the hematopoietic cell lines examined and are also down-regulated to low levels in TPA activated U-937 cells (to levels similar to AZU1), indicating responsiveness to TPA-mediated (direct or indirect) down-regulation. The level of ADN transcription in TPA-treated U-937 cells is comparable to its expression in the other cultured hematopoietic cell lines analyzed. ADN expression is not detectable in the non-hematopoietic cell line COLO 201. HL-60 cells were shown to have a pattern of APEA expression similar to U-937 cells (not shown). Mature peripheral blood granulocytes were found not to express any of the four genes of this cluster (not shown).

DNase I hypersensitive mapping of the APEA gene cluster in neutrophil elastase expressing cells.

DHS analyses were performed on a greater than 50 kb region surrounding the APEA gene-cluster. DHS analysis of more distant DNA was not performed as D. Jenne (Munich) had determined that no serine protease gene sequences were detected within 50 kb in either direction beyond these genes using PCR primer pairs for conserved serine protease gene sequences (Wong et al. 1999). During the

process of locating genes and expressed sequence tags (EST) on a cosmid contig, the gene for polypyrimidine tract binding protein (PTB) on the telomeric side of AZU1 and the human transcript Hs.24441 (Unigene designation) on the centromeric side of ADN were identified, indicating that the region encompassing the four serine protease genes represents a complete multigene locus.

An array of distinct DHS is detected at the APEA locus in U-937 cells, within a region from 15 kb upstream of the AZU1 gene to approximately 15 kb downstream of ADN. Multiple overlapping restriction enzyme series (see Fig 5.2) were used to confirm the location of each DHS. Typical blots are shown, demonstrating each of the seventeen DHS observed (Fig 5.3 A-D, and Fig 5.4). DHS-4, 5, 10, and 12 coincide with the promoter regions of AZU1, PRTN3, ELA2, and ADN, respectively (Fig 5.2 and 5.3A,B,D), consistent with a model of nucleosome exclusion resulting from binding of the basal transcriptional machinery in these regions. DHS-12 and 14 (Fig 5.3D, 5.4) appear as triplets and doublets, respectively, on some Southern blots; on others they appear as a single intense band and have each been considered to be a single DHS rather than distinct sites due to their proximity. Higher resolution examination of DHS-12 generally demonstrates three distinct bands on Southern analysis (Fig 5.3D), indicating that the DHS are located within approximately 0.5 kb. The presence of three distinct sites at this region suggests an extended region of chromatin disruption of ~0.5 kb (Fig 5.5A) or possibly the existence of three populations of cells, each with a slightly different chromatin conformation at the ADN promoter (Fig 5.5B).

DHS-6 is located within the PRTN3 gene (Fig 5.2, 5.3B), and DHS-11 appears in the 3.5 kb region between ELA2 and ADN (Fig 5.2, 5.3D, 5.4). Although analysis of nuclease hypersensitivity is not a quantitative technique, differing intensities of each DHS relative to adjacent sites on the same blot can be visualized from the Southern blots. DHS-9 is a particularly prominent site in U-937 and HL-60 cells, appearing ~ 2 kb upstream of ELA2 (Fig 5.3B). DHS-13 and 15 are very weak sites in U-937 cells,

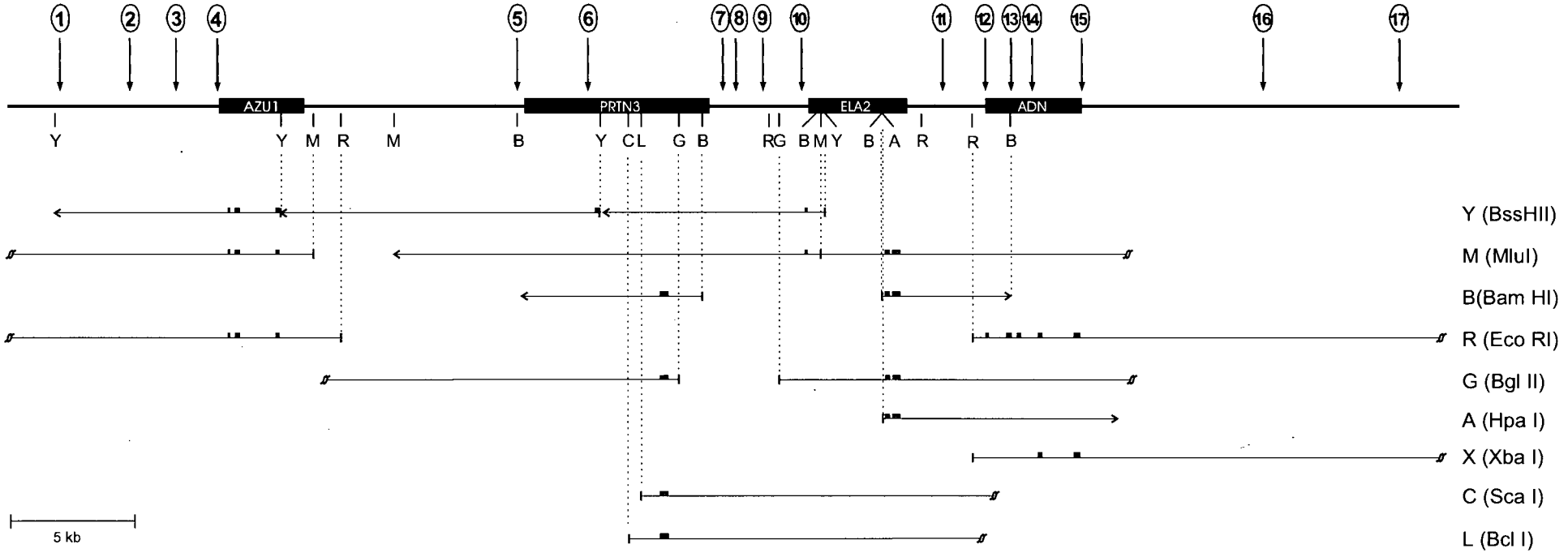

Figure 5.2- Schematic representation of regions of DNase I hypersensitivity at the APEA locus.
The locations of DHS (vertical arrows) are shown relative to a scale map of the APEA locus; the relative locations of the four genes (closed boxes, as indicated; intergenic and flanking DNA represented by horizontal lines) to the restriction sites (short vertical lines) used in the mapping of DHS at the locus are shown (A,HpaI; B,BamHI; C,ScaI; G,BglII; L,BclI; M,MluI; R,EcoRI; Y,BssHII). The regions of DNA used as probes (half-boxes) for indirect end-labeling are shown with respect to the restriction fragment used for this purpose (long arrows). Multiple analyses using different restriction fragments were used to confirm the location of the DHS in U-937 and other cell lines. Short squiggles indicate restriction fragments beyond which DHS were not clearly identified with that probe/ fragment combination or where the location of the next restriction site is unknown. The relative size of a 5 kb fragment is indicated at the lower left.

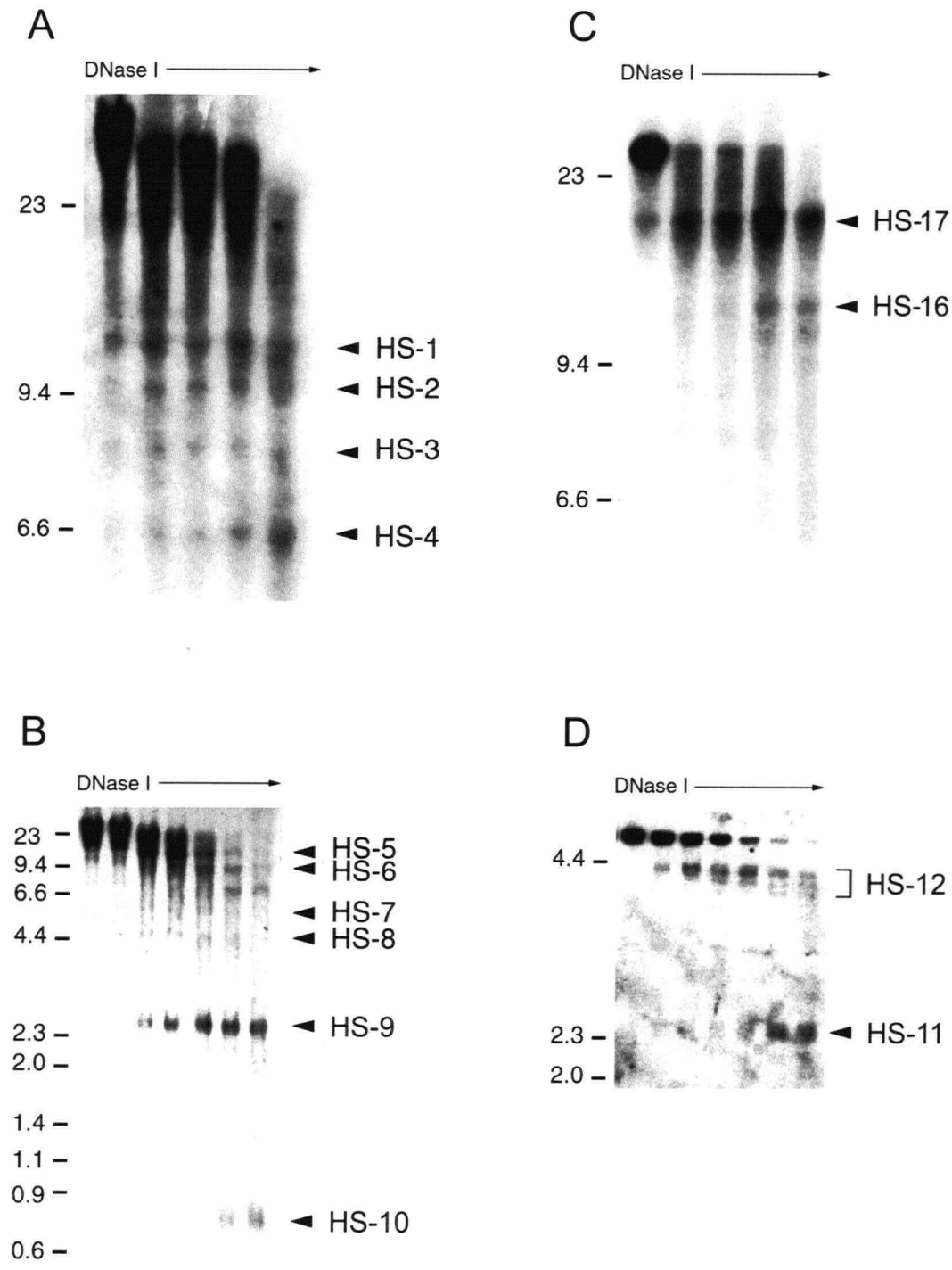
A
DNase I
23
9.4
6.6
HS-1
HS-2
HS-3
HS-4
C
DNase I
23
9.4
6.6
HS-17
HS-16
B
DNase I
23
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
HS-5
HS-6
HS-7
HS-8
HS-9
HS-10
D
DNase I
4.4
2.3
2.0
HS-12
HS-11

Figure 5.3- Analysis of chromatin structure at the AZU1-PRTN3-ELA2-ADN (APEA) locus in untreated U-937 cells.

Nuclei were prepared and treated with increasing concentrations of DNase I (0 to 5 g/mL, increasing from left to right in roughly equivalent increments of DNase I concentration in each case). The location of molecular weight DNA markers are shown on the left, in kilobases. DHS are indicated by solid arrowheads, with DHS-1 lying towards the AZU1 portion of the locus and DHS-17 at the ADN end of the locus. (A) DHS-1 to 4. DNase I treated DNA was digested to completion with EcoRI and hybridized with a PCR amplified, ^{32}P-labeled AZU1 exon IV/V cDNA probe. (B) DHS-5 to 10. DNase I treated DNA was digested to completion with Mlu I and hybridized with a PCR amplified ELA2 exon I probe. (C) DHS-16 and 17. DNase I treated DNA was digested to completion with Eco RI and hybridized with a ^{32}P-labeled ADN cDNA probe. (D) DHS-11 and 12. DNase I treated DNA was digested to completion with Bam HI and hybridized with a ^{32}P-labeled ELA2 exon IV/ V probe.

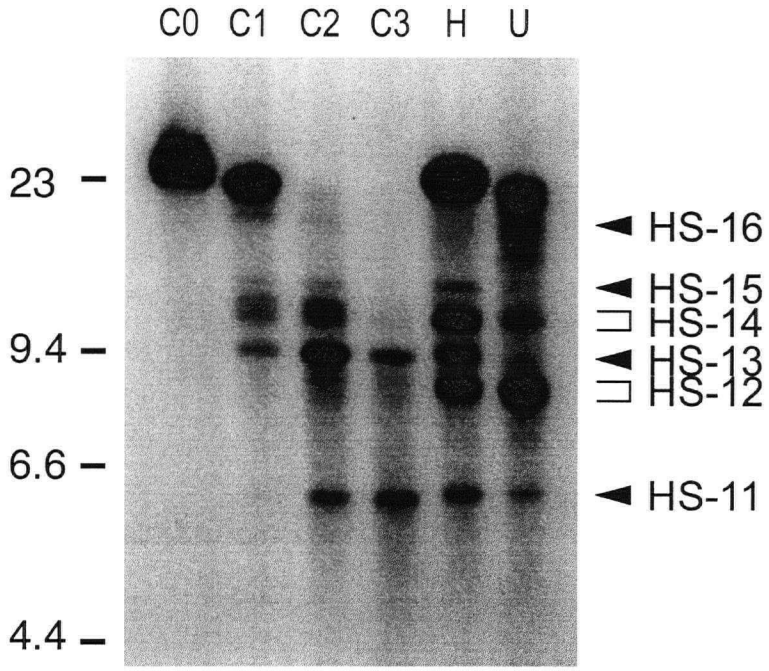

Figure 5.4-. Analysis of DHS-11 to 16 in U-937, COLO 201, and HL-60 cells.
All lanes are DNase I treated DNA digested to completion with Bgl II and hybridized with a ^{32}P-labeled ELA2 exon IV/V cDNA probe. From left to right: C0, COLO 201 DNA not treated with DNase I; C1 to C3, COLO 201 DNA from nuclei treated with increasing concentrations of DNase I; H, HL-60 DNase I treated time-point; U, U-937 DNase I treated time-point. The locations of the DNA molecular weight markers used are shown on the left, and the location of DHS-11 to 16 are indicated by arrowheads on the right.

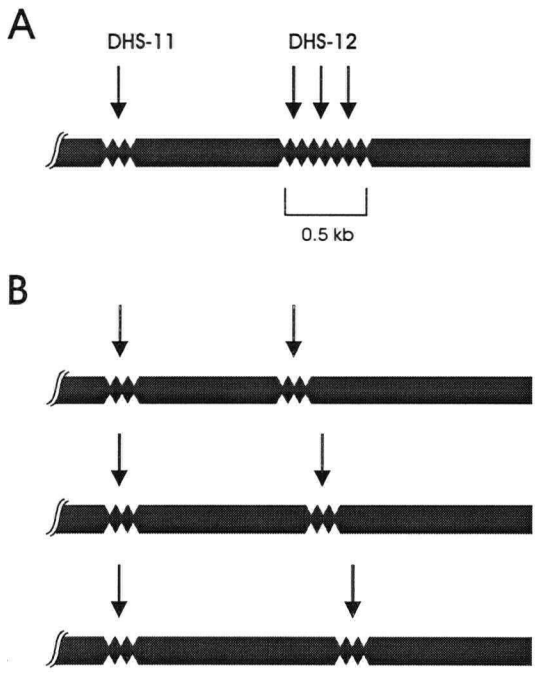

Figure 5.5- Possible configurations of chromatin at DHS-11/ 12 in actively transcribing cells.
DHS-12 is located in the proximal promoter region of the ADN gene in U-937 cells (arrows). In most blots, DHS-12 is composed of 3 distinct bands within approximately 0.5kb. Two possible configurations could account for these sites: (A) There is a large region of altered chromatin structure (jagged bar) of ~0.5 kb which is hypersensitive to nucleases, or (B) there are three populations of cells, each with a different specific location of DHS-12. Regular (non-hypersensitive) chromatin is represented by a solid bar.

appearing stronger in HL-60 cells (Table 5.1). Two sets of hypersensitive sites, DHS 1 to 4 and DHS 16 and 17 flank the locus (Fig 5.2, 5.3A and C). Although these studies are structural in nature, the conserved pattern of nuclease hypersensitivity between HL-60 and U-937 cells* in comparison to non-myeloid cells suggests that this pattern of chromatin organization is likely to be important in regulating expression of the genes at this locus.

TPA-differentiated U-937 cells possess a similar chromatin structure to non-treated, undifferentiated cells.

Treatment of U-937 cells with TPA has previously been shown to induce partial differentiation of these cells (Stockbauer et al. 1983). TPA-treatment resulted in a loose-aggregation of U-937 cells at 24 hrs, accompanied by a reduction of cell proliferation and cessation of cell division by 36 hours (see Fig 5.1). Although TPA-induced differentiation is accompanied by down-regulation of ELA2, PRTN3, and AZU1 transcription, the DHS pattern at the APEA locus in these cells is identical to that of un-treated, ELA2-expressing U-937 cells for all the identified sites in terms of position and relative intensity (Table 5.1 and not shown), indicating that change in chromatin structure at this locus is not necessary for transcriptional down-regulation of the ELA2, PRTN3, and AZU1 genes.

The absence of observable changes in chromatin organization in TPA-treated U-937 cells suggests that down-regulation of the AZU1, PRTN3, and ELA2 genes induced by TPA is likely to be a consequence of the effects of TPA on *trans*-acting factors involved in the transcription of these genes. C/EBPα and c-myb are candidate transcription factors which may be involved in TPA mediated down-

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* The DHS discovered in U-937 cells were confirmed subsequently in HL-60 cells (identifying DHS- 13, Fig 5.2) by Dr. S. Porter.

regulation of these genes. Both C/EBPα and c-myb are expressed in early myeloid cells and their levels are decreased upon differentiation. Consensus binding sites for C/EBP factors are present in the promoters of all three genes and c-myb binding sequences are present in the promoters of ELA2 and PRTN3. Both C/EBP and c-myb consensus sequences are important in ELA2 and PRTN3 promoter activity, suggesting a role for these factors in mediating transcription of these genes (Sturrock et al. 1996; Nuchprayoon et al. 1994). In particular, TPA induced differentiation in HL-60 cells has been shown to reduce c-myb levels (Boise et al. 1992). The ets factors PU.1 and GABP (ets factor family members) are also likely to be involved in transcriptional regulation at the ELA2 locus. Ets binding sites are present in the promoters of ELA2 and PRTN3, and mutation of this site in the ELA2 promoter decreases promoter function (Nuchprayoon et al. 1997). TPA induced differentiation of U-937 cells is accompanied by phosphorylation of PU.1, affecting PU.1 binding activity (Carey et al. 1996). Treatment with a PK-C inhibitor antagonizes TPA induced differentiation, indicating that decrease in ELA2 and PRTN3 gene expression following TPA treatment in U-937 cells may be mediated in part by alteration of PU.1 activity. Hence, though transcription at the locus may require an active chromatin structure, regulation by *trans*-acting factors alone may be sufficient for mediating the initial down-regulation of transcription in myeloid cells accompanying differentiation of the promyelocyte.

The myeloperoxidase (MPO) gene shares a similar pattern of expression with ELA2, being expressed at the blast, promyelocyte, and early myelocyte stages (Fouret et al. 1989) and therefore conceivably shares a similar mechanism in mediating this pattern of myeloid-specific expression. The consequences of TPA treatment at the APEA locus however, contrasts distinctly with the response of the myeloperoxidase (MPO) gene locus, one of the few myeloid-specific genes which has been well studied in detail at the chromatin level. TPA-induced granulocytic differentiation and down-regulation of MPO expression is accompanied by *loss* of three DHS in the region proximal to the 5' end of the gene (Lübbert et al. 1991; Jorgenson et al. 1991), whereas no changes in hypersensitivity were observed at any of the

sites in the APEA locus. Although there are regions of extensive similarity between the promoters of MPO and ELA2 (Takahashi et al. 1988b), the promoters of ELA2, PRTN3, and AZU1 possess a TATAA consensus sequence, whereas the promoters of MPO and the majority of other myeloid-specific gene promoters which have been studied do not. Furthermore, although MPO is myeloid-specific, its expression differs from that of the AZU1, PRTN3, and ELA2 genes in that MPO expression is not specific to the neutrophilic path of myeloid differentiation but is also expressed during monocytic differentiation (Pryzwansky et al. 1978). The significance of the differences between chromatin remodeling at the ELA2 and MPO loci in relation to potential similarities or differences between their mechanisms of transcriptional regulation is presently unknown.

Chromatin reorganization occurs during terminal granulopoiesis.

To determine whether any changes occur at the APEA locus accompanying terminal differentiation to the mature neutrophil, DHS were examined in peripheral blood granulocytes. Complete DHS analysis of the APEA locus in mature peripheral neutrophils (PMN) demonstrated the absence of any DHS (summarized in Table 5.1). Loss of DHS-1 to 17 in neutrophils indicates that extensive chromatin remodeling occurs at this locus upon differentiation of the promyelocyte to mature neutrophils. Approximately 4 cell divisions occur subsequent to the promyelocyte stage, during the myelocyte stage (Wickramasinghe and Moffat, 1971). The extensive chromatin reorganization occuring within a maximum of four cell divisions is in contrast with a previous study of the chick globin genes in which temperature-sensitive tumor virus transformed chick fibroblasts, which do not normally express the globin genes, begin to transcribe these genes (Groudine and Weintraub, 1982). Transcription is accompanied by formation of hypersensitive sites in the the absence of *de novo* RNA or protein synthesis. Raising the temperature to non-permissive levels halts gene expression; however, the hypersensitive sites are propagated for up to twenty cell divisions. These experiments indicated that 1) hypersensitive sites may be

necessary but are not *sufficient* for gene expression. (This view is in agreement with the aforementioned data, which demonstrated that TPA-induced cessation of ELA2 transcription is not accompanied by change in chromatin organization); 2) once initiated, hypersensitive sites can persist through numerous population doublings in the absence of additional stimuli and without synthesis of new proteins. This suggests that the chromatin reorganization observed at the APEA locus accompanying the 4 cell doublings during terminal granulocytic differentiation does not result from a passive erosion of the well-defined chromatin structure at this locus. An initiating factor, such as change in transcription factor profile upon terminal differentiation possibly causes this chromatin remodeling. Loss of trans-acting factor binding could conceivably allow nucleosome formation over DHS previously occupying hypersensitive sites (Felsenfeld et al. 1996). Another possibility is that condensation of the APEA locus is a consequence of the more global chromatin condensation occuring within the differentiating PMN; the condensed chromatin within the multi-lobed nucleus of the mature neutrophil is well documented. Expression of a myeloid-specific protein, MENT (myeloid and erythroid nuclear termination stage-specific protein), specifically in terminally differentiated erythrocytes and neutrophils has recently been reported (Grigoryev and Woodcock, 1998). MENT is associated with heterochromatin in neutrophils and may be in part responsible for chromatin condensation in these cells. Reorganization of chromatin and associated changes in transcriptional activity of the genes at the ELA2 locus may therefore be a result of widespread condensation of chromatin in neutrophils mediated by MENT and/ or other factors.

Differential chromatin structure at the APEA cluster of genes in cells which do not express neutrophil elastase.

An important issue in understanding the potential relevance of chromatin organization at the APEA locus is whether this is a cell-type specific phenomenon. For instance, does the chromatin structure at the "active" (AZU1, PRTN3, ELA2 expressing) locus correlate with expression of these genes?

Non-myeloid hematopoietic cells and a representative non-hematopoietic cell line were used to examine this issue in more detail: Complete DHS analyses of the entire locus was repeated in each of the cell lines examined; the data are summarized in Table 5.1. In the non-myeloid cell lines studied, only a small subset of the DHS observed in U-937 cells exist. DHS-1 appears only in the erythroid line, K-562, and DHS-2 appears in both K-562 and COLO 201 cells. Unlike the hematopoietic lines, COLO 201 also carries DHS-11 and 12. DHS-16 appears in COLO 201 and also in HUT-78 cells but is not present in HL-60 cells. The other sites, DHS-3 to 10, appear exclusively in the early myeloid cell type. DHS-17 is present in COLO 201 cells and in all the hematopoietic lineages studied, but not in mature neutrophils and is thus common to all the hematopoietic and non-hematopoietic lineages. DHS-13 to 15, which appear weakly in U-937 cells, are also present in all the cell lines examined, again, with the exception of mature neutrophils.

The general loss of DHS in non-expressing cells of hematopoietic or non-hematopoietic origin strongly suggests a functional role for these sites in regulating transcriptional activity in myeloid cells. In particular, the specificity of DHS-3 to 10 to both U-937 and HL-60 cells indicates that they may be involved in positive transcriptional regulation of these genes. DHS-3 to 10 are furthermore localized to the immediate vicinity of the AZU1, PRTN3, and ELA2 genes, which differ in their pattern of gene expression from ADN. DHS-13 to 15 are present in both myeloid and non-myeloid cell types, which together with their location in the ADN gene, suggest a role in mediating positive regulation of this gene. Appearance of the altered chromatin organization at the ELA2 locus in early myeloid-committed cells but not in the closely related erythromyeloid cell line K-562 further suggests that the active chromatin

Table 5.1 Differential organization of DHS at the APEA locus in myeloid versus non-myeloid cells.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
U-937	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	↓	↓	↓	⇩	⇩
+ TPA	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	↓	↓	↓	⇩	⇩
HL-60	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩		⇩
COLO		⇩									⇩	↓	⇩	⇩	⇩	⇩	⇩
HUT													⇩	⇩	⇩	⇩	⇩
Jurkat													⇩	⇩	⇩		⇩
K562	⇩	⇩											⇩	⇩	⇩		⇩
PMN													nd				

The presence of DHS-1 to 17 (columns) are indicated by open arrows or thin arrows (extremely weak or barely distinguishable site) in each of the cell lines studied (rows), (+TPA, TPA-treated U-937 cells; PMN, peripheral blood granulocytes; nd, not determined). The presence of each DHS was confirmed by overlapping DHS analyses as for Fig 5.3. Figure on top is provided for reference (see Fig 5.2 for notes).

structure at this locus is formed subsequent to the CFU-GEMM stage at which these lineages are related, in myeloid-committed cells. An alternate explanation is that this pattern of hypersensitivity existed at that stage but was actively remodeled upon commitment to the erythroid lineage. Both possibilities point to a direct or indirect active rearrangement of chromatin at the ELA2 locus that parallels cell type specific transcriptional activity at this locus.

Although the AZU1, PRTN3, and ELA2 genes can be down-regulated in myeloid cells in the presence of an active chromatin conformation, it is possible that an "inactive" chromatin conformation characterized by the absence of DHS seen in non-myeloid cell types is important in preventing aberrant expression of these genes. This would allow transcriptional silencing of the AZU1, PRTN3, and ELA2 genes even in cell types where C/EBPα, c-myb, PU.1, or other *trans*-acting factors involved in transcription of these genes are present. Since TPA induced transcriptional down-regulation in U-937 cells is not accompanied by change in the active chromatin conformation of the APEA locus, loss of the DHS accompanying terminal neutrophil differentiation likely occurs subsequent to transcriptional down-regulation and may possibly serve as an additional barrier against aberrant expression of these genes. The biological significance of such a stringent control of transcription of these genes is substantiated by the destructive nature of neutrophil elastase and proteinase 3 and their potential contribution to inflammatory-related disease. Specific down-regulation of expression at the APEA locus mediated by inactivation of transcriptionally permissive chromatin structure presents a potential means through which this repression may be implemented.

The physical proximity, common spatial and temporal pattern of expression, homologous gene structure, and coordinate down-regulation of AZU1, PRTN3, and ELA2 suggest that the mechanisms governing the transcriptional regulation of these genes are related and exist in a common transcriptional regulatory domain. If regulation of transcription of these genes are indeed governed by common *cis*-acting elements, then it is conceivable that an insulatory element exists between the AZU1, PRTN3 and

ELA2 genes on one side, and ADN gene on the other, as ADN exhibits a pattern of expression which is distinct from the other genes in the region. Studies in other model systems have demonstrated the existence of such insulatory elements which can serve to delimit transcriptional units (Geyer, 1997). The gypsy element of *Drosophila* is one of a few extensively studied examples of an insulator element. The gypsy element binds to the *su(Hw)* protein and can block the ability of an enhancer to activate transcription from a downstream promoter (Cai and Levine, 1995); this insulator also protects against chromosomal position effects (Roseman et al. 1993). The most distal 3' DHS, DHS-17, is present in all the cell types examined except mature neutrophils. As no additional sites were detected beyond this DHS, it is possible that DHS-17 serves an analogous role to the chicken ß-globin constitutive DHS at the 5' domain border, a boundary element, delimiting the gene domain and shielding it from the effects of neighboring chromatin organization and from other transcriptional regulatory influences (Chung et al. 1993).

Functional characterization of the DHS at this locus both *in vitro* and in transgenic animals should demonstrate their associated functions, some of which are likely to be involved in mediating the stringent, high-level expression of ELA2, PRTN3, and AZU1 in granulocytic precursors *in vivo*. Chapter 6 describes experiments performed to study selected DHS flanking the ELA2 gene.

Chapter 6- A Cis-acting Element Important in Transcription of the Neutrophil Elastase Gene in the Presence of Chromatin*

RATIONALE AND HYPOTHESIS

As discussed, the expression of neutrophil elastase is highly restricted both spatially and temporally. ELA2 transcripts are found exclusively in myeloid cells (above), and expressed within a narrow window in early granulocyte differentiation, predominantly at the promyelocyte stage (Fouret et al. 1989; Takahashi et al. 1988a). This strict pattern of gene expression is necessitated by the potential toxicity of the gene product in heterologous tissues and by the important role of timing of expression on granule targeting (Chapter 2 and Arnljots et al. 1998). The mechanisms underlying promyelocyte-restricted gene expression are not known. Of the myeloid-specific genes, only the mechanisms governing chicken lysozyme gene expression have been examined in thorough detail, and the lysozyme locus is considered a paradigm in the study of vertebrate gene expression regulatory mechanisms (reviewed in Sippel et al. 1992). Lysozyme transcriptional regulation is not representative of genes specific to the granulocyte path of myeloid differentiation however, as it is expressed largely in macrophages and its expression increases with differentiation of these cells. Granulocyte-specific expression has been recently attained in vivo but the mechanisms governing this expression pattern have not been reported for any of these systems: An 18.8 kb genomic DNA encompassing the FcγRIII gene was shown to reconstitute the correct granulocyte expression pattern of the gene (Li et al. 1996). This expression pattern was conferred by a ~5 kb 5' and ~5 kb 3' flanking region but the elements within this region conferring this activity were not more precisely identified. A 6 kb cathepsin G gene containing ~2.5 kb 5' flanking

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* Data presented herein form the basis for a manuscript, A Chromatin Dependent Enhancer Important in Directing Expression of the Neutrophil Elastase Gene, by **Edmond T. L. Wong**, Susan D. Porter, and C. Blake Gilks. (submitted), 1999.

and 0.8 kb 3' flanking DNA also demonstrated myeloid specific expression (Grisolano et al. 1994); however, the elements regulating this expression pattern were not examined. An MRP8 (migration inhibitory factor-related protein 8) transgene was also shown to induce myeloid specific expression in the bone marrow, spleen, and blood; again, the responsible sequences conferring this expression pattern were not further characterized.

The current understanding of granulocyte-specific expression derives largely from examination of proximal promoter elements in granulocyte-specific genes using transient transfection. Such studies have demonstrated a propensity for myeloid-specific promoters to contain consensus binding sites for

Table 6.1 Myeloid-associated transcription factors

Gene	Transcription factor binding sites	References
neutrophil elastase	PU.1, c/EBPα, PEBP2/CBF (e.g. AML1), Myb, GABP	(Srikanth and Rado, 1994; Nuchprayoon et al. 1994; Nuchprayoon et al. 1997)
Proteinase 3	PU.1, c/EBPα, Myb	(Zimmer et al. 1992)
Azurocidin	PU.1, c/EBPα, Myb	(Zimmer et al. 1992)
CD11b (CR3)	Sp1, PU.1	(Chen et al. 1993)
FcγRIII (CD16)	Sp1, PU.1, Ets, GATA, NF-IL-6	(Feinman et al. 1994)
myeloperoxidase	Sp1, PU.1, PEBP2/CBF	(Nuchprayoon et al. 1994)

Notes: Representative myeloid-specific genes and potential transcription factor binding sites found in their proximal promoter regions. This list of myeloid specific genes and associated binding sites is not exhaustive. Many additional myeloid-specific genes exist and possess some additional though less common sequences, including AML1, c-Myc, EGR-1, C/EBPβ, etc.

C/EBP, Ets, AP-1, and Sp1 transcription factors (TABLE 6.1). None of the mentioned transcription factors however, are specific to myeloid cells, suggesting that they may act in concert to mediate tissue-specificity; however, this hypothesis remains to be proven. It is possible that other factors are also involved in regulating myeloid specificity. A plausible mediator of myeloid-specific transcription is the MZF-1 transcription factor, a zinc finger protein that is notable in that it is the only known myeloid-specific transcription factor, indicating a potential role in mediating myeloid cell specific transcription (below and Bavisotto et al. 1991; Morris et al. 1995).

Characterization of proximal ELA2 gene regulatory elements has similarly identified potential trans-acting factors involved in regulation of the gene (Srikanth and Rado, 1994; Srikanth and Rado, 1998). In common with promoters of most genes expressed in myeloid cells, the ELA2 promoter harbors transcription factor binding sites for the PU.1, Sp1, c/EPB and c-myb transcription factors (Fig 4.4). The same consensus binding sequences are also found in the mouse Ela2 promoter (Nuchprayoon et al. 1994), where c/EBP, c-Myb, and PU.1 have been demonstrated to cooperatively activate transcription at the Ela2 promoter in a non-myeloid cell line (Oelgeschläger et al. 1996), demonstrating a role for these factors in mediating transcription of the gene. While mouse and human neutrophil elastase promoters have myeloid specific activity in transient transfection assays, the ability of these regions to direct correct spatial and temporal expression of neutrophil elastase *in vivo* had not been determined. As demonstrated in Chapter 2, the proximal promoter region which has been well examined from the perspective of mediating cell-type selective expression in transient transfection assays is not capable of mediating the high levels of endogenous expression of neutrophil elastase observed in neutrophil precursors.

Hypothesis:

Elucidation of the chromatin organization at the ELA2 locus through the identification of DNase I hypersensitive sites (DHS) will demonstrate regions of transcriptional regulatory activity involved in mediating correct spatial and temporal expression of the gene (see also Chapter 5).

Specific Aim: Characterize the transcriptional regulatory activity of DHS-9, 10, 11, and 12 in ELA2-expressing myeloid cells in the absence and presence of chromatin organization.

In the preceding chapter, the chromatin structure of the NE locus was described through identification of DHS at this locus. Numerous studies have associated DHS with regions of functional activity, in replication, transcription, and recombination, as described. In consideration of their proximity to the ELA2 gene and their specificity to myeloid cells, DHS-9, 11, and 12 were selected for further functional characterization. DHS-10 coincides with the ELA2 promoter, which is encompassed within the HNE transgene. Its function has thus already been described in Chapter 2. DHS-9, 11, and 12 lie outside of the region used in HNE transgenic mice, and have not previously been characterized functionally. In the previous chapter, the active chromatin organization at this locus in ELA2 transcribing cells suggests that chromatin organization is important in regulating gene expression at this locus. Here, DHS-9 is identified as a chromatin-dependent enhancer of transcription important in permitting transcription of a stably transfected reporter gene.

METHODS

Constructs used for transfection

The plasmids used here are also shown schematically in Figure 6.2A and B. Briefly, to prepare pDHS-10S GL3, a region from the transcription initiation site of human NE up to -153 bp was amplified

from genomic DNA via PCR using the primers 5'-GGCTGAAGCTTCGGGGTCTCTGCCCC-3' and 5'-CCGGTACCGAGCCAATCCAGCGTCTT-3' and then ligated to the Bgl II and Kpn I sites, immediately upstream of the luciferase gene in the pGL3 Basic reporter vector (Promega). This DHS-10S PCR-amplified promoter region was completely sequenced and matched the reported sequence. pDHS-10L GL3 is similar to pDHS-10S GL3 except that up to 1167 bp of upstream DNA was prepared by ligating a HNE1KB/ HAN1B primer-pair PCR amplified fragment into the Bgl II and Hind III sites of pGL3. The 272 bp region adjacent to the transcription initiation site was sequenced and confirmed to match the published sequence. The pDHS-9 GL3 and pDHS-11/12 GL3 vectors were based upon the pDHS-10L GL3 vector. DHS-9 was cloned from the Azi-13 cosmid. To confirm the location of DHS-9 relative to the EcoRI site in the 5' DNA flanking ELA2 (-1418), MluI/ EcoRI cut cosmid Azi-13 DNA was electrophoresed alongside MluI-digested U-937 DNA treated with DNase I for detecting DHS. ELA2 exon I probed Southern analysis of this DNA revealed that DHS-9 is located distal to the EcoRI site relative to ELA2 (not shown; see Fig 5.2). A Bgl II subclone that was shown to hybridize to a PRTN3 exon 5 cDNA probe in colony hybridization (to prevent possible confusion with similar sized subclones) was derived from cosmid Azi-13 to facilitate the further subcloning of DHS-9. A 1.4 kb BamHI/ EcoRI fragment located between -2238 and -1413 bp relative to the NE transcriptional initiation site, containing the entire DHS-9 was therefore subcloned from the Bgl II subclone into the BamHI/ Sal I site of pDHS-10L GL3 to make pDHS-9 GL3. pDHS-11/12 GL3 was constructed by ligating the downstream 5.4 kb BamHI fragment from cosmid Azi-13, encompassing DHS-11,12, to the BamHI site of pDHS-10L GL3 in the endogenous orientation relative to the gene and was distinguished from inappropriately subcloned fragments via colony hybridization with an ELA2 exon 4/5 cDNA probe. DNA was purified with anion-exchange chromatography (Qiagen) or with a large scale alkaline lysis procedure followed by PEG-precipitation (Sambrook et al. 1989).

Transient transfections

Transient transfections were performed with purified, supercoiled DNA. Optimization of electroporation resulting in transient expression identified a sensitive response of U-937 cells to electroporation field strength (V/cm) (Fig 6.1), demonstrating an optimal transfection efficiency at 300 V/cm (using a Bio-Rad Gene Pulser system). Two pmol (~5 to 10 μg) of reporter construct were electroporated with 5 μg pCMVβgal (Clontech) and pBS carrier DNA (Stratagene) into 1.4×10^7 U-937 cells from log-phase culture in 0.5 ml RPMI at 300 V and 960 μF in 0.4-cm electroporation cuvettes. Cells were then incubated at 4 °C for 15 min and returned to culture medium for 8 h, washed twice in phosphate-buffered saline, and lysed with Reporter Lysis Buffer (Promega Corp.). Luciferase activity was quantified with luciferase substrate (Promega) and β galactosidase activity was determined with a chemiluminescent substrate based assay (Tropix or Galacton), both according to the manufacturer's directions using single-photon detection in a liquid scintillation counter. Each data point used in the calculation of statistics was derived from independently quantitated and electroporated DNA. The calculation of luciferase activity for each construct, per data point, is represented by the equation (cpm^s_{luc}, sample luciferase activity (in counts-per-minute, cpm); cpm'_{luc}, background luciferase activity; $cpm^s_{\beta gal}$, sample β-galactosidase activity; $cpm'_{\beta gal}$, background β-galactosidase activity):

$$\text{luciferase activity} = (cpm^s_{luc} - cpm'_{luc}) \cdot (cpm^s_{\beta gal} - cpm'_{\beta gal})^{-1}$$

The arithmetic mean of the resulting data points for each construct is taken and expressed as a percentage of pGL3-Promoter luciferase activity.

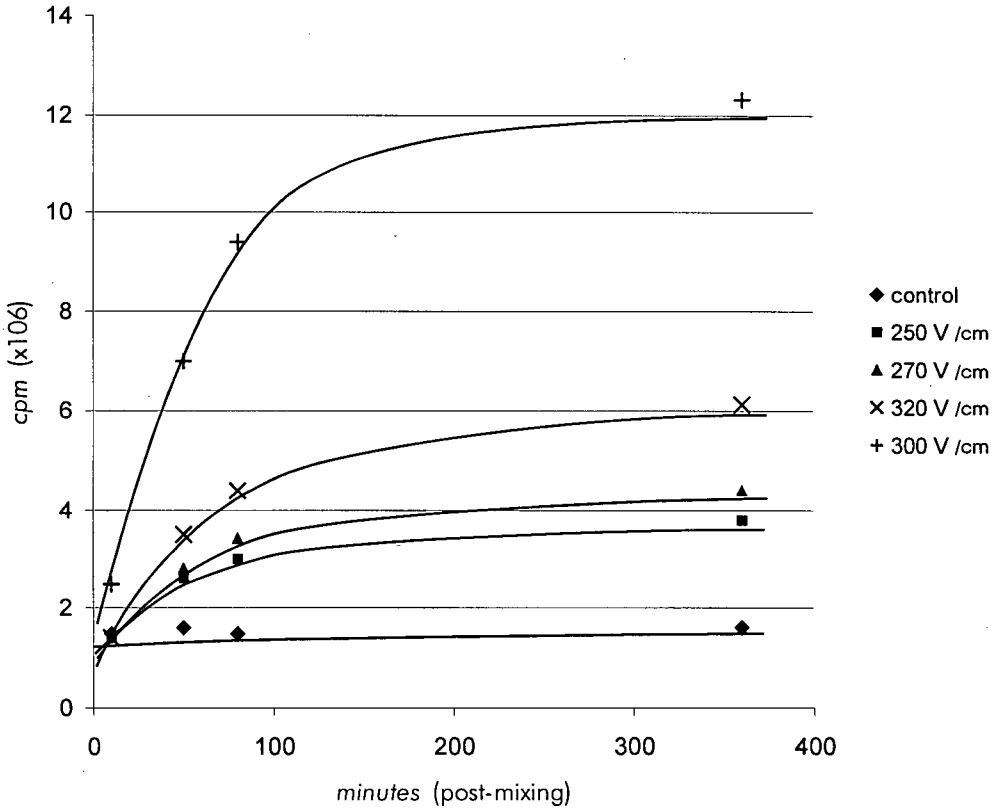

Figure 6.1- U-937 cell transfection optimization.
Electroporation field strength (V/cm) had the most pronounced effect on transfection efficiency: pCMV-βgal plasmid was transfected at the indicated voltages into U-937 cells (see Methods). Luciferase activity (cpm, single-channel photon counting on a scintillation counter) is shown as a function of time, at 10, 50, 60, and 360 minutes post-electroporation. Expression was maximal at 8 hours (not shown), 300 V.

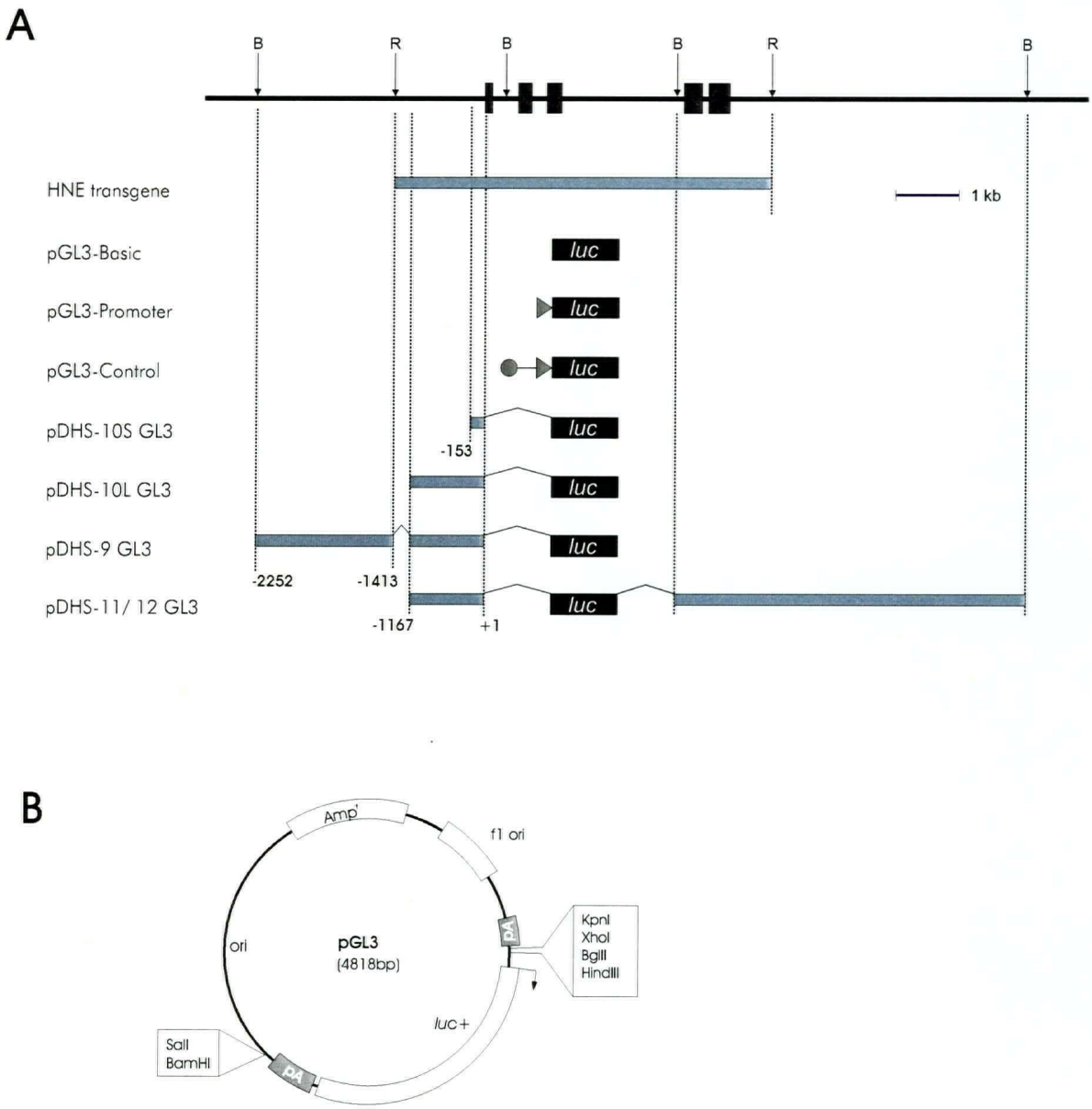

Figure 6.2- Diagram of ELA2 DHS-9 to 12 reporter gene constructs.
(A) A partial restriction map of the ELA2 gene is shown on top (B,BamHI; R,EcoRI; closed boxes depict ELA2 exons). Shaded boxes represent regions of the ELA2 gene used in each construct and are on the same scale as the restriction map (extended to its corresponding region with a dotted line; numbers indicate distance from ELA2 transcription initiation site (+1)). The SV40 promoter (triangle) is used to drive expression of pGL3-Promoter, and the SV-40 enhancer (circle) is attached in pGL3-Control; the luciferase gene (*luc*) is indicated (not to scale); constructs are based on (B), the pGL3 luciferase reporter vector (Promega): *luc*+, modified firefly luciferase gene; pA,poly(A) signal; Amp^R, gene conferring ampicillin resistance in E.coli. Unique restriction sites used in subcloning are indicated.

Stable transfections

To establish suitability of G418 or hygromycin for use as a positive selection marker, dose-response curves comparing concentration of G418 or hygromycin B (Clontech) to viability were established for U-937 cells. Cell death was visualized by fragmentation of nuclei and trypan blue exclusion. U-937 cells exhibited a very high tolerance to G418, up to >1600 μg/ml; however, cell death did not appear at a clear concentration or time-point, with a partial population of the cells much more susceptible to the chemical than others. Tolerance of cells appeared to range from 800 to >1600 μg/ml even at two weeks. Tolerance to addition of hygromycin to 100 μg/ml was comparable to that of untreated cells, but at ≥150 μg/ml, cell death in the entire population was distinct by day 7 of culture. Stable transfections were performed in a similar manner to the transient protocol except that transfected DNA was linearized with Sal I and co-transfected with 5 μg PGK-Hyg (PGK promoter driven hygromycin resistance expression vector) instead of pCMVβgal and no carrier DNA was used. Cells were then passaged for two doubling periods in RPMI supplemented with 10 % fetal calf serum and then selected in the same media containing 150 μg/ml hygromycin B (Clontech). Cells were pelleted every ~2-5 days to remove cellular debris and to replace the hygromycin and culture medium. Non-pPGKhyg transfected cells, mock-transfected cells, and diluted, untransfected cells were used as controls for monitoring the integrity of hygromycin treatment for all transfections. Three separately transfected cultures of each construct were pooled to minimize the variability of expression which may result from position effects of integration. Relative transgene copy was determined through hybridization of the NcoI/ XbaI fragment of pGL3 to NcoI/ XbaI digested, Southern blotted DNA from stably transfected cells for each sample and quantitated with computer-assisted densitometry of the bands. Luciferase activity per transgene copy was then obtained for each pool by normalizing against relative transgene copy numbers and against total protein content for the sample, which was determined with the Bradford assay, and is represented by the following equation:

$$\text{luciferase activity} = (\text{cpm}^{s}_{luc} - \text{cpm'}_{luc}) \cdot [(\text{total protein})(\text{relative copy number})]^{-1}$$

The luciferase activity for each construct is expressed as a percentage of the construct with the highest level of expression.

RESULTS AND DISCUSSION

The myeloid-specific DHS-9 mediates expression in the presence of chromatin organization.

As discussed, chromatin organization has important effects on gene transcription, and the studies in Chapters 2 and 5 emphasized the relationship between chromatin structure and transcription of ELA2. Additionally, it appears that the previously studied ELA2 proximal promoter region is not sufficient to mediate transcription in vivo; previous studies had not elucidated the mechanisms which allow for ELA2 transcription in the presence of chromatin. To identify additional cis-acting elements in the NE locus which are capable of activating NE gene transcription, the potential transcriptional regulatory properties of the DHS regions adjacent to the NE gene were assessed. Transient transfection of the minimal NE promoter-driven reporter vector pDHS-10S GL3 (Fig 6.2) into U-937 cells confirmed a substantial increase in expression above control levels exhibited by the promoterless pGL3 Basic vector (Fig 6.3). The 5'-flanking region was extended to encompass DNA to 1167 bp upstream of the transcription initiation site in pDHS-10L GL3, encompassing the previously described heterologous enhancer element REP53 (Yoshimura et al. 1994). Transient transfection of this region revealed that the REP53 enhancer (see Fig 4.4) in the pDHS-10L GL3 construct has no significant effect upon transcriptional activity in U-937 cells, which goes against the suggestion by Yoshimura et al. that REP53 has a relevant role in mediating expression of ELA2. As non-expressing cell types were used by the Yoshimura/ Crystal group, the enhancer activity may be an artifact of their model system. Addition of DHS-9 or DHS-11/ 12 to the pDHS-10L GL3 construct had no significant effect on transcription mediated by DHS-10S (the -153 minimal promoter), implying that the maximal transcriptional activity within 1167 bp region of DNA flanking the 5' end of ELA2 is conferred predominantly by the minimal promoter region to -153. This finding is contrary to the initial findings of the Rado group, but is largely consistent with their subsequent findings (Srikanth and Rado, 1994).

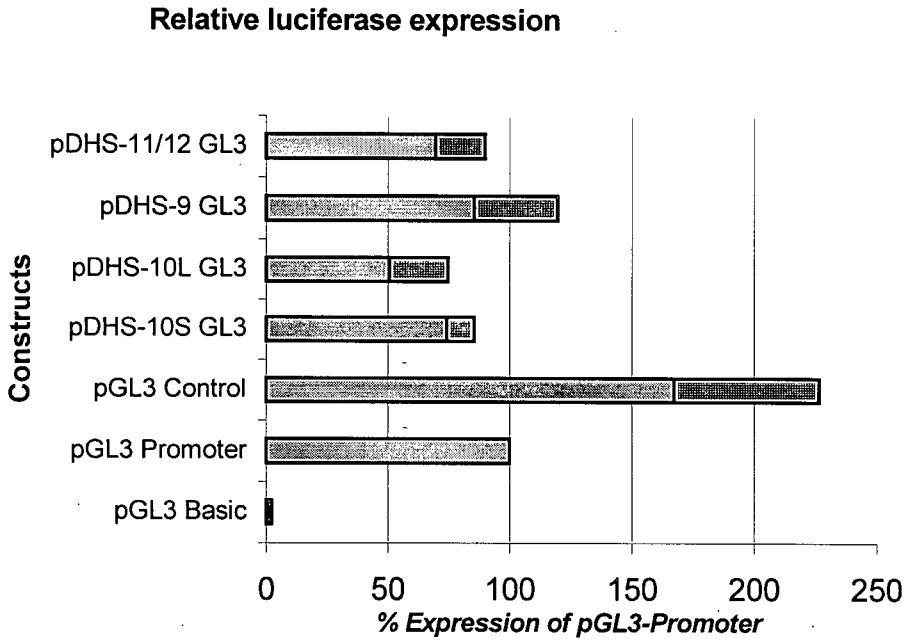

Figure 6.3- Functional analysis of the ELA2 5′ and 3′ DHS by transient transfection.
This figure summarizes the ability of the myeloid-specific DHS-9 and 10, and of DHS-11 and 12 to enhance transcription from the native ELA2 promoter (pDHS-11/ 12, pDHS-9, pDHS-10, p[-153]GL3) or of the heterologous SV-40 promoter (pGL3 Promoter, pGL3 Basic). U-937 cells were transiently transfected with the pGL3-based luciferase reporter vectors shown on the *y*-axis. Luciferase activities (x-axis) were represented as a percentage of pGL3-Promoter (100%) after correcting for differences in transfection efficiency. *n*= 4 or 5 for all experiments and the bars show the standard deviation of the mean in each case. Data points are also shown in tabular form below.

	n	Average	SD
pGL3 Basic	4	1.35	0.79
pGL3 Promoter	4	100.00	0.00
pGL3 Control	4	167.50	58.95
pDHS-10S GL3	4	74.00	11.34
pDHS-10L GL3	4	50.50	23.97
pDHS-9 GL3	4	85.25	34.17
pDHS-11/12 GL3	5	69.59	20.16

To examine the transcriptional regulatory activity of these same regions in the context of chromatin, stable transfections into U-937 cells were performed. Stable transfection of pDHS-10S GL3 resulted in no luciferase activity beyond control levels (Fig 6.4). This was in agreement with our observations with TgHNE mice (Chapter 2) that -1416 bp of 5' flanking DNA was unable to drive transcription in vivo but contrasted with the transient assay results. Stably transfected pDHS-10L GL3 exhibited a slight increase in reporter gene transcription above background levels which were barely detectable in this system and which by itself is unlikely to account for the high level of endogenous expression in myeloid cells. Stably transfected pDHS-9 GL3 however, was expressed at levels more than 1000-fold greater than the expression mediated by pDHS-10L GL3. DHS-11/ 12 also increased luciferase expression, but at more modest levels, being approximately 50-fold over that exhibited by pDHS-10L GL3.

The ability of the minimal NE promoter region in pDHS-10S GL3 and of the extended promoter region in pDHS-10L GL3 to drive transcription in U-937 cells in transient transfection assays confirms that they possess an intrinsic ability to activate gene expression in myeloid cells. This observation contrasts with the transgenic TgHNE model, which demonstrated that DNA within –1416 bp of the ELA2 5' flanking DNA are not capable of driving NE expression *in vivo*. Stable transfection of the same reporter constructs to U-937 cells demonstrated that the DHS-10S and DHS-10L regions are unable to induce reporter expression when stably transfected, consistent with the hypothesis that lack of NE transgene expression in TgHNE mice is a consequence of chromatin-mediated repression.

In U-937 cells, the DHS-9 element drives transcription even in the presence of chromatin. In transient assays however, neither DHS-9 nor DHS-11/ 12 exerted significant influences upon transcriptional activity, suggesting that DHS-9 enhances ELA2 transcription by derepressing chromatin-mediated suppression of transcription at the locus rather than directly enhancing the kinetics of gene transcription. The myeloid-specific appearance of DHS-9 and DHS-11/ 12 at the NE locus strongly

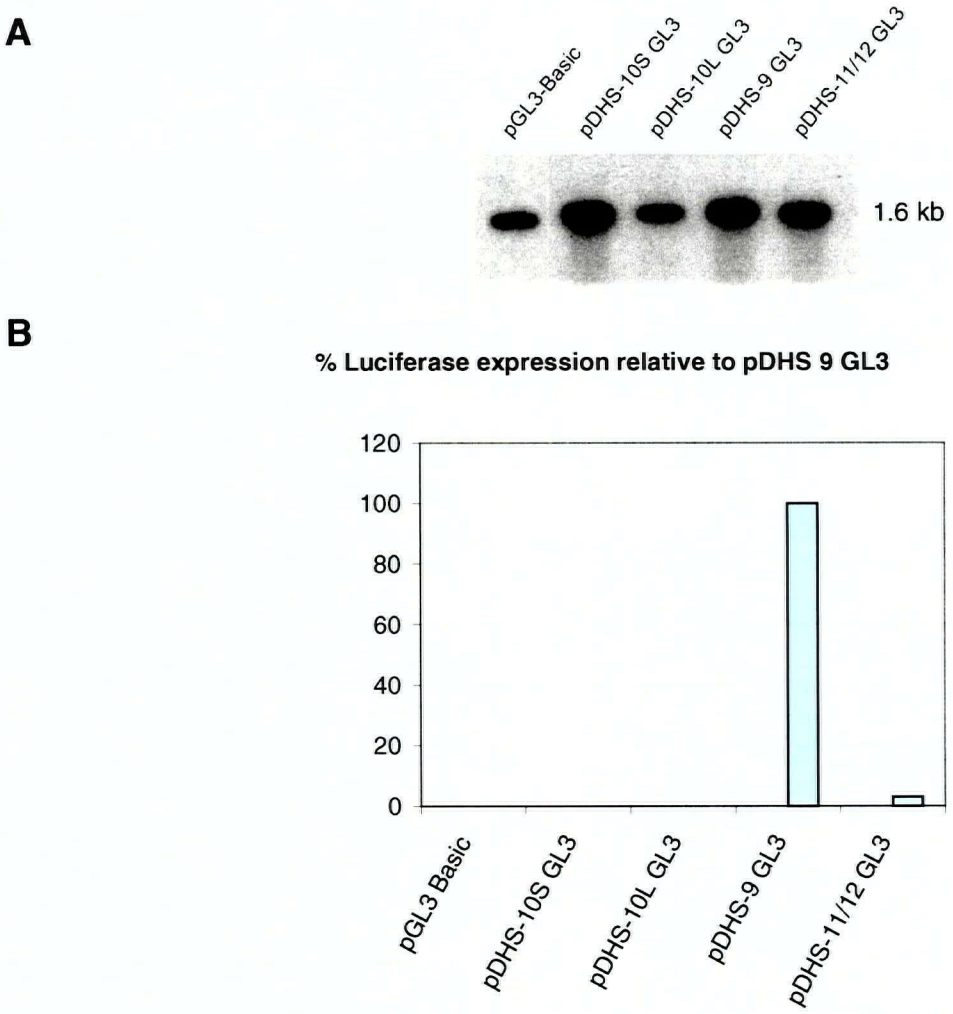

Figure 6.4- Functional analysis of ELA2 transcriptional regulatory activity in stably transfected U-937 cells.

A. Southern analysis of NcoI/XbaI-digested genomic DNA from the indicated stably transfected cells used for transfection with luciferase-specific probe (refer to Methods).

B. The level of transcription conferred by stably integrated ELA2 flanking DHS, DHS-9, 10, 11, or 12, on the native ELA2 minimal promoter-driven reporter gene (*luc*) in U-937 cells is shown as a percentage of maximal observed transcription (*y*-axis). Luciferase activity was normalized by correcting for construct size, relative integrated gene copy-number, and total cellular protein. Stable transfection was obtained by co-electroporation with a pPKG-Hyg plasmid encoding resistance to the drug hygromycin. Data points are also shown in tabular form below.

	% luciferase expression relative to pDHS9 GL3
pGL3 Basic	0.00
pDHS-10S GL3	0.00
pDHS-10L GL3	0.06
pDHS-9 GL3	100.00
pDHS-11/12 GL3	3.00

suggest that these elements function in a myeloid specific manner, suggesting a possible means through which expression of the NE gene may be restricted to myeloid cells. The observation that the DHS-10 (–1167) region was unable to enhance expression in the transient assays is distinct from the previous finding that REP53 acts as a transcriptional enhancer. This is perhaps due to our use of myeloid cells rather than heterologous cell types. Stable transfection of this region however, demonstrates a slightly increased level of transcription which was at the threshold of detection in our system. This modest increase in the level of transcription would probably not impact significantly upon transcription in vivo as demonstrated by the TgHNE mice, which carry this region in the transgene.

Chromatin dependent enhancer activity

Chromatin dependent enhancer activity such as that exhibited by DHS-9 is a feature which has not been well examined in its own right, though its importance can be inferred from other studies (above). One of a few specific examples of chromatin-dependent enhancer activity was a recent report indicating that transient transfection of a CD34 promoter-driven reporter gene to which a DNA fragment containing DHS 2, 3, and 4 from the murine CD34 locus have been appended enhances expression by ~ 1.2 to 2.5-fold, whereas expression is increased by greater than 75-fold when stably integrated (May and Enver, 1995). A similar observation was made with the stably transfected human β-globin HS3 in MEL cells (Collis et al. 1990). An *in vitro* enhancer assay on chromatin-assembled plasmid DNA supports these observations, as a 50-fold increase in relative enhancer activity is demonstrated in comparison to naked DNA (Workman et al. 1991). Although the effect of enhancer activity in the presence of chromatin is often overlooked in lieu of simpler transient assays, the effect of chromatin on enhancer activity appears to be an essential feature of certain enhancers. The traditional definition of an enhancer as a cis-element capable of up-regulating gene expression in a position and orientation-independent manner should perhaps be expanded for eukaryotes to distinguish between the transcriptional activity that can be observed in transient assays from that which occurs in the presence of chromatin organization of the inte-

grated/ endogenous gene. The relationship between the dominant chromatin-opening activity of the 'LCR' region of β-globin and chromatin-dependent enhancers such as DHS-9 was specifically examined in one study (Jackson et al. 1996); chromatin-dependent activity and enhancer function are related key features between the two and appears to be a separable feature of the LCR. It is conceivable that DHS-9 may share other properties characteristic of a typical LCR such as the ability to confer position independent and copy-number dependent expression of a transgene. Whether chromatin-dependent elements or LCRs exist in all or most loci however, still remains to be determined.

DHS-9 contains multiple MZF-1 consensus binding sites.

Analysis of the 800 bp DHS-9 element reveals that it contains multiple consensus binding sites for MZF-1, Myb, AP-4, and Ikaros-2, which appear at frequencies 4, 4, 10, and 4 times more frequently than predicted for a random sequence of the same size (TRANSFAC (Heinemeyer et al. 1998); core and matrix consensus sequence similarities >90%) (Fig 6.5 and Appendix). Mutational analysis and affinity for the respective transcription factors would confirm these sites. A DNA sequence homology search* indicated several >30 bp regions from unrelated loci sharing >95% identity; however, these DNA sequences were associated with large cosmid clones which have not yet been extensively described (not shown).

The prevalence of Myb consensus binding sequences in DHS-9 and its previously described significance in myelopoiesis suggests a role for this factor in mediating DHS-9 activity. The Myb proto-oncoprotein is expressed at high levels in early hematopoietic lineages and is required for definitive hematopoiesis in the mouse embryo (Mucenski, 1991). Myb expression is down-regulated upon differentiation of early hematopoietic cells. Constitutive expression of c-myb however, has been demonstrated to

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* gapped BLASTn search

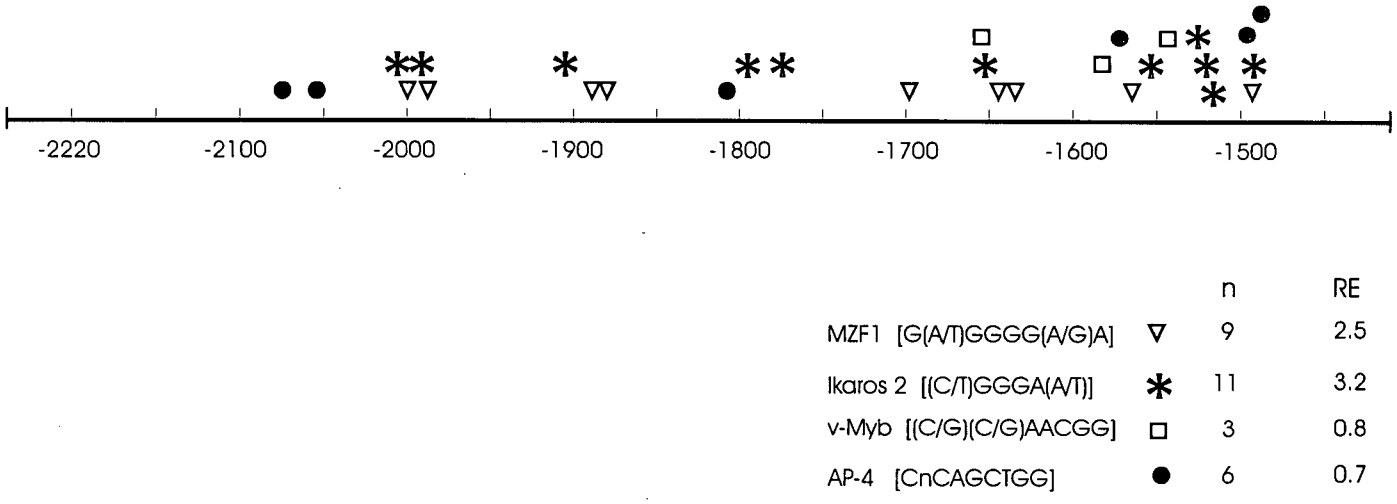

Figure 6.5- Schematic map of transcription factor consensus binding sequences in the DHS-9 region represented in pDHS-9 GL3.
Sequencing analysis was performed on the 5' ELA2 region containing DHS-9. The locations, to scale, of the transcription factor consensus binding sequences are indicated (see inset) relative to the distance, in base pairs, from the ELA2 transcription initiation site. Potential transcription factor sites shown are selected on the basis of notable over-representation of that sequence within the pDHS-9 GL3 insert and restricted to sites with >90% similarities in core and matrix sequences to the consensus sequence (see inset, in brackets); (n, observed frequency; RE, expected frequency for a random DNA sequence of the same size).

block transition from the promyelocyte to the metamyelocyte stage (Bies et al. 1995). At the NE promoter, Myb has been demonstrated to cooperatively activate transcription in the presence of C/EBP, PU.1, and GABP (Oelgeschläger et al. 1996). The direct correlation between expression of NE and Myb and the presence of multiple Myb consensus binding sites in DHS-9 in myeloid cells is consistent with a role for Myb in mediating the positive transcriptional regulatory effects of DHS-9.

MZF-1 consensus binding sequences also appear four times more frequently than predicted for a segment of DNA this size. The MZF-1 zinc-finger protein is the only known myeloid-specific transcription factor, and is expressed primarily in the myelocyte and metamyelocyte stages of neutrophilic development. The exact physiological role of MZF-1 has not been determined; however, aberrant expression of this transcription factor has been demonstrated to affect erythromyeloid development of ES cells and has also been shown to inhibit c-myb and CD-34 promoter-mediated transcription (Perrotti et al. 1995). The positive correlation between MZF-1 expression and myelopoiesis and the over-representation of MZF-1 consensus sequences in DHS-9 suggests the possibility that MZF-1 contributes to the regulation of NE expression during differentiation.

The relative abundance of AP-4 consensus binding sites is of particular interest as the AP-4 site is found in the simian virus 40 (SV-40) transcriptional enhancer (Mermod et al. 1988). The SV-40 enhancer region has been shown to coincide with a DHS, appearing on electron microscopy as a nucleosome-free region. Recently, a deletion-analysis of this region demonstrated that the AP-4 consensus binding site is important in conferring nuclease sensitivity to the region (Friez et al. 1999), suggesting that the AP-4 activator protein may be at least in part responsible for establishing the disrupted chromatin at this region. The abundance of AP-4 sites in DHS-9 presents a plausible mechanism for the formation of DHS-9, which may inherently contribute to its chromatin-dependent enhancer activity.

The Ikaros-2 consensus sequence appears more frequently than expected. However, the Ikaros family of transcription factor is lymphoid-specific (Georgopoulos et al. 1992) and would thus be unlikely to function in the activity of the DHS-9 element. A similar transcription factor binding a similar

consensus sequence in myeloid cells may exist, but none have yet been identified. Nonetheless, the over-representation of this consensus sequence and the observation that Ikaros is a hematopoietic specific transcription factor suggests that a related transcription factor may act in this region.

The presence of multiple consensus transcription factor binding sites likely results in an increased probability of transcription factor binding as a result of increased exposure of these sites irrespective of translational or rotational positioning. In theory, the presence of two consensus binding sites would double the probability of transcription factor binding, three sites would triple the potential affinity, and so forth. An observation supporting this view is that mutation of transcription factor binding sites of a β-globin promoter correlates inversely with transcriptional activity and DHS formation (Boyes and Felsenfeld, 1996).

Summary of Part II

Despite numerous studies which have focused entirely on the relevance of proximal promoter regions in mediating ELA2 expression, the studies presented in Chapter 2 demonstrated that additional factors are involved in the expression of this gene in vivo. Here, by establishing a profile of DHS at the ELA2 locus in myeloid cells which express ELA2 and comparing this to the chromatin in non-expressing cells, a distinct pattern of chromatin organization was observed and is furthermore specific to cells in which ELA2 and the adjacent, evolutionarily conserved genes AZU1 and PRTN3 are expressed, together suggesting an important role of chromatin organization in NE gene expression. The identification of ADN in the vicinity of these genes raises the additional question of how this gene is differentially regulated. Functional examination of DHS flanking the ELA2 gene led to the conclusion that DHS-9 is an important element in permitting high-levels of expression in the presence of chromatin. DHS-9 was demonstrated to carry an over-representation of AP-4, Myb, MZF1, and Ikaros 2 consensus binding sequences, indicating a likely role of the respective transcription factors in mediating the functional activity

of DHS-9 enhancer activity. The ability to perform structure/ function correlations to study myeloid (and other) gene expression in the near future will be greatly facilitated by the imminent completion of the genome sequencing project*, which, in addition to facilitating cloning and restriction site identification, etc., would allow large segments of DNA within loci with similar expression patterns to be compared (for example, a great deal of effort was involved in the aforementioned work in restriction mapping of the 3' end of the Azi-3 cosmid, in determining the presence of repetitive elements, etc., but can now be readily derived from recently posted sequencing data†). Together with the further evaluation of other myeloid-specific genes in the context of chromatin, a more refined understanding of the in vivo transcriptional regulation of myeloid, and particularly granulocyte-restricted gene expression should soon become apparent.

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* Expected completion in 2003 (NIH Press Release, 15 Mar 1999)

† i.e., LLNL Genome Center cosmid R33516 (GenBank AC004799)

Possibilities for Future Research

Part I of this thesis described experiments conducted to study the potential role of NE in emphysema. Historically, technical limitations have limited the construction of an accurate model to test the potential role of NE in this disease. Although the advent of transgenic technology has provided the potential for developing such a model, attempts to do so have remained elusive due at least in part to an inadequate understanding of NE gene expression mechanisms. Part II addressed the possibility that chromatin structure plays an integral role in NE gene expression, and these structural and functional studies led to the identification of DHS-9, an element likely to be important in mediating the expression of NE in vivo. DHS-9 functions only in the context of stable chromatin integration, suggesting a role in upregulating NE gene expression by alleviating chromatin-dependent transcriptional repression.

A number of potential future experiments based upon these observations are possible, depending largely upon the interest of the researcher. From the view-point of NE in emphysema, a candidate regulatory element has been identified which could be appended to an NE transgene to facilitate NE over-expression. A more tactical approach would be to use the cathepsin G promoter region, which has been recently used to direct myeloid-specific expression (Grisolano et al. 1994). Other myeloid-specific regulatory elements can be examined concurrently to maximize the possibility that one will work. The possibility remains however, that a DHS-9 driven transgene or a cathepsin G promoter-driven NE transgene is toxic due to low levels of mis-expression, as seen with the haptoglobin promoter-driven TgHpNE construct. Retrovirus-mediated gene transfer of NE into the lung might circumvent this problem by avoiding potential misexpression in non-pulmonary or non-myeloid tissues. Experiments determining the susceptibility of NE deficient mice to smoking- or LPS instillation-, etc.-induced emphysema are already underway in Stephen Shapiro's laboratory (St.Louis) and should answer many of the existing questions regarding the role of NE in emphysema. Since there is still controversy as to whether the macrophage or neutrophil is more important in induction of emphysema (Chapter 1), development of a neutrophil or

monocyte lineage-specific knock-out and testing its susceptibility to airspace enlargement would be equally if not more relevant. A number of existing models exist, which could be tested immediately: C/EBPβ disrupted mice for instance have impaired macrophage function and PU.1 deficient animals lack the myeloid and lymphoid lineages.

From the perspective of NE gene expression, an obvious line of experimentation would be to elucidate the mechanism of DHS-9 mediated, chromatin-dependent enhancer activity. Conventional studies using electrophoretic mobility shift assays to determine whether myeloid-specific factors (e.g. MZF1) bind to DHS-9 would be informative. DNase I foot-printing assays would further pin-point specific regions which are likely to be involved in trans-acting factor binding, revealing the elements involved. A bolder approach would be to co-transfect AP-4, MZF-1, Ikaros-2, and/or c-Myb expression vectors with a DHS-9 linked reporter vector into various myeloid and non-myeloid cell lines to determine a possibility for direct involvement of one of these factors in mediating DHS-9 enhancer activity. Ideally, DHS-9 will be introduced into transgenic mice, linked to a reporter gene driven by the ELA2 promoter. This experiment would allow for the visualization of expression in the developing animal and in adult tissue and will indicate the in vivo significance of DHS-9 in directing gene expression. From the perspective of DHS-9 chromatin-dependent mechanisms, an interesting experiment would be to stably transfect a DHS-9 linked reporter construct into U-937 or 32Dc13 (a murine early myeloblast cell line capable of differentiating to the promyelocyte stage and capable of complete differentiation) cells, expanding different clonal populations, and determining 1) position-dependence of expression 2) copy-number dependence of expression 3) association between level of expression and recreation of the DHS-9 site through DNase I hypersensitivity assays. In addition to further studies of DHS-9, a number of other potentially interesting DHS at the locus can be examined using assays such as those described in Chapter 5.

Now that large cosmid clones of the locus are available, these can be used to generate transgenic animals. An initial impediment in my studies was the absence of any indication of where functional ele-

ments may exist, whereas identification of the human β-globin locus LCR for instance, benefited from the characterization of the various thallasemias. Generation of transgenic mice carrying large cosmid fragments would facilitate the identification of functionally important sites when used together with the DHS mapping reported here. In particular, the overall effect of DHS-9 in mediating LCR-like activity (dominant enhancer activity conferring position independent, copy-number dependent expression of a transgene) could be determined. General sensitivity at this locus can also be examined if desired, to confirm a chromatin domain boundary. Since ADN is expressed differently from the other genes in the locus despite its proximity, it will be interesting to determine whether there is any correlation between expression of ADN and general chromatin organization, and if so, what is responsible for segregating the ADN chromatin domain from that of the other three genes in the locus. The aforementioned studies should advance our current understanding of NE gene expression.

Nomenclature

α1AT	alpha-1-antitrypsin
α2MG	alpha-2-macroglobulin
ADN	adipsin (complement factor D) gene, adipsin
APEA	the AZU1-PRTN3-ELA2-ADN genes
AZU1	azurocidin gene, official gene symbol
BAL	brochoalveolar lavage
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenical acetyl transferase [gene]
CFU	colony forming unit
CFU-GEMM	granulocyte-erythroid-megakaryocyte-macrophage CFU
CFU-GM	granulocyte-macrophage CFU
COPD	chronic obstructive pulmonary disease
cpm	counts per minute
CTSG	cathepsin G gene, official gene symbol
DHS	deoxyribonuclease I hypersensitive site
DNase I	deoxyribonuclease I
ECM	extracellular matrix
EDTA	disodium ethylene diamine tetraacetate
Ela2	mouse neutrophil elastase gene, official gene symbol
ELA2	human neutrophil elastase gene, official gene symbol
ELF	epithelial lining fluid
FMLP	formyl-methionyl-leucylphenylalanine
H&E	hematoxylin-eosin
HpNE	haptoglobin promoter-neutrophil elastase transgene construct
HSV-TK	herpes simplex virus thymidine kinase [gene]
hyg	hygromycin B phosphotransferase gene
kb	kilobase pair
LPS	lipopolysaccharide
Mb	megabase pair
mM	millimolar
MoE	macrophage elastase
NE	neutrophil elastase
NO	nitric oxide
PCR	polymerase chain reaction

PEG	polyethylene glycol 8000
PGK	phosphoglycerate kinase [promoter]
PMN	polymorphonuclear leukocyte
Prtn3	mouse proteinase 3 gene, official gene symbol
PRTN3	human proteinase 3 gene, official gene symbol
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SLPI	secretory leukoprotease inhibitor
SP-C	surfactant protein C
TIMP	tissue inhibitor of metalloproteinase
TPA	12-o-tetradecanoyl-phorbol-13-acetate

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Appendix

List of oligonucleotides

Oligonucleotide	Sequence
AZU1	5′- CGGGATCCATGACCCGGCTGACAGTCC -3′
AZU2	5′- CGGAATTCGCAGCATCAGGTCGTTCAGGTT -3′
BGR1	5′- TAGAATTCAGAGACCCCGGAGCCCCAGCCC -3′
BGR2	5′- GTAAGCTTCAGAGAATGTTTTATTGTGCCAG -3′
BGR3	5′- GAGGATCCAACTGCCATCATACTGAAGGTA -3′
BGR4	5′- GAGAATTCTTGGTTGGTCTTGCCTCTGGA -3′
BGR5	5′- CCCCAGCAGCAAGGCCGGCA -3′
BGR6	5′- CGGGATCCCGTGGGGCCGCGCTCGCCGG -3′
GL2	5′- CTTTATGTTTTTGGCGTCTTCCA -3′
HAN1	5′- GGGAGATCTGGTCTCTGCCCCTCCGTGCC -3′
HAN1B	5′- GGGCTGAAGCTTCGGGGTCTCTGCCC -3′
HAN5	5′- CCGGTACCGAGCCAATCCAGCGTCTT -3′
HNE1KB	5′- TCTCTCTCGAGCAGCCCTGCCAGA -3′
HNEEX5A	5′- GGGGATCCGGCAGCCCCTTGGTCT -3′
HNEEX5B	5′- AGGAATTCTCAGTGGGTCCTGCTGG -3′
Hpp1	5′- AGAAGTGAGCTAGTGGC -3′
MNE1	5′- TGGTGTCTTTACTGGAGGTG -3′
MNE2	5′- CGAAGCCATAATGACCACCT -3′
MNE3	5′- ATGGCCCTTGGCAGACTATCCA -3′
MNE4	5′- CTTCTAGGCGACGTGTCTCTTC -3′
MNE5	5′- ACTCTAGAGACCTTCTCTGTGCAGCGGATC -3′
NEX1H3	5′- GGGCTAAGCTTCCGGGGTCTCTGC -3′
PR3EX4A	5′- GCGGATCCGCCCAGCCAACCTCAGTGC -3′
PR3EX4B	5′- ACGAATTCGATGCCGGCCTTGCGGCGA -3′

DNase I hypersensitive site 9 (DHS-9) DNA sequence analysis

DNA sequence consensus matches with a core similarity of 1.00 and a matrix similarity of >0.900 were determined using the Transfac V2.2 database (see Chapter 6, Results). The consensus binding sequences are: AP-4 (AP4), CWCAGCTGG; Ikaros 2 (Ik2), YGGGAW; Myb, SYAACGG; MZF-1 (MZF), GNGGGGA; with standard nomenclature for the nucleotides (and W=A/T, Y=C/T, and S=C/G). See Heinemeyer et al. (1998) for additional details.

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826 bp

          1234567890 1234567890 1234567890 1234567890 1234567890

-2238     ggatcctacg attataattt tacagaggag gaaactgagt cacggagagt

-2188     ttatttcagg ggcctgaagt tacacagtgg ccaaatggcc acaccagaac

-2138     caggatatga acccagcgtc ggcacactta ataatgactg cacgtgagaa

                AP4                       AP4
-2088     tgtagtatac agctggtgct caataaatgc agctgacgcc aagtctgatc

                                                  Ik2 MZF
-2038     tccgtgctgg aggccaagcc ccttccaaag atacggcgtc ccccacccct

             Ik2 MZF
-1988     gaaatccccc caccccgac ttgaaacagg gctgtgtaga cagtaggcgc

                                                 Ik2
-1938     ttactaagtg cactgtggtg atgttaaggc agcaaggtgg gaaccctgcc

              MZF  MZF
-1888     aaaaggggag gggagaaggc agggtagtgt gtgtctgatt aagctgagga

                                       AP-4              Ik2
-1838     cagggcagac atggactgag cgcctgctgt gtgccaggct ctgggagggc

                        Ik2
-1788     agggccattg tctccctaac ccgagagcca tgggggtcca cttgcctgtg

                                                    MZF
-1738     gtcacgtcag gactccagcc tggcccaggc tctgcgtgtc cccgggtgcc

                                          Myb      Ik2    MZF  MZF
-1688     ctcgccccgc ctattcctgg agacaggccc gttggttccc ttcccctccc

-1638     cttgtcctgg agccaggagg acgttggttc ttgcgacagc cttggcccgg
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                Myb AP-4                MZF     Ik2       Myb
-1588      ccgttgcagc tggaacatcg tgggggagat gggaagagga acggggcccg

                                Ik2  Ik2   Ik2     AP-4 Ik2   AP-4
-1538      gagcccgggg ctgggtcctg ggaatccctt tcccgcagct gggactccag

             MZF
-1488      ctcccctgcc agttcctcca ggcggaagcc ctcaggcttg gtcctcactc

-1438      cagcctcccg gcctggacag gaattc
```