ABSTRACT

In a eukaryotic cell, the nuclear and cytoplasmic compartments are segregated by the nuclear envelope, a highly ordered, supramolecular complex, consisting of the outer and inner nuclear membranes, the nuclear pore complexes and an underlying nuclear lamina. The nuclear lamina is a karyoskeletal structure composed of a fibrous meshwork of proteins and lies subjacent to the inner nuclear membrane on the nucleoplasmic face. It provides an architectural framework to the nuclear envelope, thereby conferring structural integrity to it, and is known to play an important role in nuclear envelope assembly and disassembly during mitosis. Recent studies suggest that the interaction of the nuclear lamina with chromatin contributes to the three-dimensional organisation of the interphase chromatin and might also influence crucial nuclear processes such as DNA replication and transcription.

The nuclear lamina is formed by the polymerisation of a class of intermediate filament proteins known as lamins, which in higher vertebrates are of two kinds - the B-type lamins, which are expressed constitutively in all somatic cells and the A-type lamins, consisting of lamin A and lamin C, which are expressed only at later stages of embryonic development. Lamins A and C have been shown to arise by the alternate splicing of the same gene. Several studies have established that A-type lamins are expressed only in differentiated cells and are not detectable in early embryos or in undifferentiated embryonal carcinoma cell lines such as PCC-4, F9 and P19. The expression of the mammalian lamin A gene has been shown to be primarily under transcriptional control, with maximal levels of lamin A transcripts being observed in actively dividing, differentiated cells including a number of cultured cell lines and fetal hepatocytes, and falling to barely detectable levels in quiescent cells like adult hepatocytes. Although studies on the differential expression of lamins have been carried out in several cell types, the mechanism of transcriptional regulation of the A-type lamins has not been investigated.

The work described in this thesis was undertaken to elucidate the molecular mechanism of regulation of the rat lamin A gene. Towards this end, a genomic clone comprising the 5' upstream promoter region of the rat lamin A gene was isolated and characterised. In vitro and in vivo experiments were carried out to identify the proximal promoter sequences and characterise the regulatory factors responsible for promoter activity. The activation of the lamin A gene in actively dividing, differentiated cells was studied using HeLa cells. Putative
factors involved in its subsequent downregulation as cells cease to divide and become quiescent were identified by a comparison of promoter-specific interactions in fetal and adult hepatocytes. The undifferentiated PCC-4 embryonal carcinoma cells which show a lack of lamin A expression were studied in an attempt to gain insights into the requirements for silencing of the gene in embryonic cells.

The thesis has been divided into seven chapters and the contents of each chapter are briefly described below.

Chapter I is the introductory chapter and presents a brief account of the progress of studies on nuclear lamins. An overview of the information available on the structure of the nuclear envelope, its assembly and disassembly during mitosis, and the structure, organisation and function of the nuclear lamina has been given. This is followed by a description of the structure and classification of the various lamin types and a review of studies on the differential expression of A-type lamins. This chapter also includes a discussion on the mechanisms of eukaryotic gene regulation, a description of the components and functioning of the eukaryotic transcription machinery and the role of transcription activators, with specific reference to the Sp1 and AP-1 family of transcription factors. Towards the end of this chapter, the background and objectives of this study are discussed.

Chapter II records the source of chemicals and materials used as well as the methodology employed during the course of this study. It includes a description of the routinely used recombinant DNA techniques, DNA sequence analysis, primer extension analysis of RNA, PCR, preparation of nuclear extracts from cultured cells and tissues, electrophoretic mobility shift assays, DNase I footprinting analysis and transient transfection assays.

Chapter III describes the cloning and characterization of the 5' proximal promoter of the rat lamin A gene. Screening of the rat liver genomic library in lambda DASH vector with a probe corresponding to the 5' region of the rat lamin A cDNA led to the isolation of a positive clone, 6F, carrying an 11 kb insert. Several fragments spanning the entire insert were subcloned into the pUC18 vector and mapped with various restriction enzymes. A 2.2 kb subclone encompassing the ATG codon at the translational start site and 2 kb of upstream sequence was sequenced and analysed for the presence of putative transcription factor binding sites using the TRANSFAC database. A GC box, TATA-like motif and three AP-1 sites were identified. A primer extension analysis of RNA showed that the transcription start site mapped
to 203 bases upstream of the ATG codon. The TATA box was found to be located at -33, the GC box at -101, and the three AP-1 sites at -7, -424 and -1677 with respect to the transcription initiation site.

In Chapter IV, the strategies used to study the activation of the lamin A gene in HeLa cells (used as a model for differentiated, actively dividing cells) and an analysis of the results obtained have been presented. Functional analysis of the lamin A promoter to identify the minimal promoter region was carried out by luciferase reporter assays, wherein several 5' deletion constructs of the 2 kb lamin promoter were cloned into the pGL3 vector and transfected into HeLa and NIH 3T3 cells. Deletion of the two AP-1 sites at -1677 and -424 did not affect the promoter activity significantly, suggesting that they are unlikely to play a major role in promoter activity, but removal of a GC box at -101 resulted in a 4 to 6-fold decrease in promoter activity. The smallest fragment tested, -60/+78, containing the TATA box and the AP-1 motif at -7 (which could not be deleted without affecting transcription) retained 15% of the activity. This suggested that the GC box and the AP-1 motif might be important for lamin A promoter function. A site-directed mutagenesis of the GC box and AP-1 motifs was performed and functional activity of the mutants was checked by luciferase reporter assays. The GC box/AP-1 double mutant showed a 60% loss in activity, confirming that these sites are essential for promoter activity. Electrophoretic mobility shift assays (EMSAs) of separate oligos encompassing the GC box and the AP-1 motif at -7 with HeLa nuclear extracts resulted in the formation of specific DNA-protein complexes at these motifs, as confirmed by competition with excess of unlabelled specific, non-specific and mutant competitor oligos. Supershift analysis with antibodies to the c-Fos and c-Jun proteins (both of which comprise the AP-1 complex) and to Sp1, Sp3 and Sp4 (members of the Sp1 family of transcription factors) indicated that the complex formed at the AP-1 motif was composed of c-Fos and c-Jun and the complexes formed at the GC box were composed of Sp1 and Sp3 transcription factors. This data was supported by DNase I footprint analysis of the -146/+75 promoter fragment, wherein the region corresponding to the GC box was protected by HeLa extracts as well as purified Sp1, and a DNase I hypersensitive site was seen close to the AP-1 motif, both of which were abolished when these two sites were mutated. In addition, the -43 to -60 region corresponding to a GT box was found to be protected. EMSAs of oligos spanning the GT box and a TCCTCC motif were carried out with HeLa extracts. Specific complexes composed of the Sp1 family of transcription factors were formed at both these
sites. Since the GC box/AP-1 double mutant still retained 40% of promoter activity, it is plausible that other motifs such as the GT box could also be contributing to promoter activity.

In Chapter V, an in vitro experimental approach was used to study the events contributing to the downregulation of lamin A expression as actively dividing fetal hepatocytes develop into quiescent adult hepatocytes. The binding of regulatory factors to the lamin A promoter was compared in adult and fetal hepatocytes by carrying out DNase I footprinting analysis and EMSAs. DNase I footprinting analysis of the -146/+75 promoter fragment revealed an extended protected region downstream of the transcription initiation site in adult hepatocyte extracts but not in fetal hepatocyte extracts. Results from EMSAs and UV crosslinking experiments suggested that the +3/+28 sequence could bind to proteins of apparent molecular masses 62 and 48 kDa in adult hepatocyte extracts. EMSAs with the AP-1 motif indicated that a specific complex comprised of c-Jun and c-Fos could be formed only in fetal hepatocyte extracts. On the other hand, in adult hepatocyte extracts, a region upstream of the AP-1 binding site appeared to be bound to a protein of apparent molecular mass 54 kDa upon UV crosslinking. EMSAs with oligos spanning the GC box, GT box and TCC motif indicated that specific complexes composed of Sp1 and Sp3 could be formed at these sites in both adult and fetal hepatocyte extracts.

Chapter VI describes the results obtained from the in vitro and in vivo functional analysis of the lamin A promoter in PCC-4 embryonal carcinoma cells, which were used as a model to study the factors contributing to silencing of the lamin A in an undifferentiated cell line. A functional analysis of the 2 kb promoter fragment by luciferase reporter assays indicated that the lamin A promoter exhibited considerable activity even in PCC-4 cells. EMSAs and DNase I footprinting experiments with PCC-4 extracts showed that the Sp1 family of transcription factors could form strong, specific complexes at the GC, GT and TCC sites, although at the AP-1 site only weak affinity complexes were formed. These results indicate that the embryonic silencing of the lamin A gene is not due to the absence of an activator or presence of a repressor in embryonic cells, but may be due to the adoption of a higher order inactive chromatin structure, that is not attained in transient transfection assays with promoter fragments. Hence it may be necessary to study the embryonic activation of the promoter in a cellular chromatin context.

In Chapter VII, the results of the earlier chapters are discussed in terms of a putative model for regulation of the lamin A gene. Furthermore, the current status and future prospects of the field in the context of this study are reviewed.

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