CHAPTER 7

CONCLUSIONS
The present study was aimed at gaining an insight into the mechanism of transcriptional regulation of the rat lamin A gene. This involved the isolation and characterisation of a genomic clone encompassing the 5' upstream region of the lamin A gene, followed by a functional analysis of promoter fragments in cultured cells to delineate the proximal promoter and *in vitro* studies on DNA-protein interactions at specific motifs. Analysis of the lamin A 5' proximal promoter has indicated a role for several *cis*-regulatory elements and the corresponding *trans*-acting factors in the modulation of promoter activity, with distinct patterns of DNA-protein interactions being observed with proliferating and quiescent cells.

An essential component of this study has been the design of an appropriate strategy to isolate a genomic clone spanning the lamin A promoter. At the time this work was initiated, the genomic organisation of the human lamin A locus and only about 200 bp of its putative promoter sequence were known (Lin and Worman, 1993). The complete sequence of the 2.2 kb *Sac* I segment of the rat lamin A promoter was analysed and found to show 72% homology to a longer fragment of the human lamin A promoter (Lin and Worman, 1997), and 95% homology to the mouse lamin A promoter (Nakajima and Abe, 1995). As a detailed functional analysis of the promoter had not been carried out in any species, the rat lamin A promoter was subjected to a deletion analysis using reporter gene constructs transfected into two mammalian cell lines, HeLa and NIH 3T3. The smallest fragment which showed substantial promoter activity was the -146/+75 segment, and this was designated the lamin A proximal promoter.

The lamin A proximal promoter has been shown to harbour three distinct sites, the GC box, GT box and TCC motif at -101, -55 and -119 positions respectively, for binding of the ubiquitously-expressed Sp1 family of transcription factors (see Fig. 7.1). *In vitro* studies have provided evidence for the specific interaction of these motifs with Sp1 and Sp3 proteins from nuclear extracts of HeLa and PCC-4 cells as well as adult and fetal hepatocytes. *In vivo* functional analysis of the promoter has indicated a requirement of the GC box for promoter activation which was supported by site-directed mutagenesis experiments.

Specific binding of c-Fos and c-Jun transcription factors at the conserved AP-1 motif (located close to the transcription initiation site) at -7 was detected in HeLa nuclear extracts. Extracts from adult and fetal hepatocytes showed distinct profiles, with specific interactions being observed in fetal hepatocytes, but adult hepatocytes displaying a lack of c-Fos and c-Jun...
Fig. 7.1. Summary of DNA-protein interactions at the lamin A proximal promoter. The TCC, GC and GT motifs which interact with Sp1 and Sp3 proteins in HeLa, PCC-4 and hepatocyte extracts, and the AP-1 motif which shows specific interactions with c-Fos and c-Jun proteins in HeLa and fetal hepatocyte nuclear extracts are highlighted and underlined. Regions spanning -19 to -8, +3 to +27, +32 to +48, +54 to +68 and -107 to -129 involved in specific interactions with adult hepatocyte extracts are double underlined in bold. The TATTAG sequence is underlined and the transcription initiation site is highlighted and marked as +1.
binding to the AP-1 motif. PCC-4 extracts showed only weak affinity interactions at this motif. The importance of this AP-1 site in promoter activation was demonstrated by site-directed mutagenesis of the AP-1 motif.

In addition to the above conserved motifs, in vitro experiments identified two other regions involved in DNA-protein interactions in adult hepatocytes: (i) a long stretch of sequence immediately downstream of the transcription start site extending from +3 to +72, and (ii) a short sequence just upstream of the AP-1 motif between -19 and -8. Preliminary experiments indicated that these regions were bound by proteins of 62 and 48 kDa, and 54 kDa respectively. As binding of these proteins to the above regions was not discernible in either HeLa or fetal hepatocytes, they might be specifically involved in downregulation of lamin A expression in adult hepatocytes. Analysis of the lamin A promoter sequence downstream of the transcription initiation site and upstream of the AP-1 motif, in order to gain a clue to the identity of the proteins interacting with these sequences, did not reveal consensus sites for known repressors. As the downstream region had a high GC content, other GC-rich negative regulatory elements such as those of the c-myc gene and the lysozyme gene silencer were considered. However, these sequences bind to a large repressor protein CTCF of apparent molecular mass 130 kDa (Burcin et al., 1997) unlike the proteins identified in this study. Another prospective candidate that has been proposed to play an important role in maintaining the quiescent state of hepatocytes by controlling growth regulatory genes is the CAAT-enhancer-binding protein (Mischoulon et al., 1992). This protein is unlikely to play a direct role in downregulating lamin A expression as its binding motif CCAAT was not present in this promoter.

In studies with PCC-4 cells, which do not express lamin A transcripts, the transfected lamin A promoter construct exhibited considerable activity. In vitro studies showed specific binding of Sp1 transcription factors at the proximal promoter and weak interactions at the AP-1 motif. Furthermore, no interactions with putative repressors were detected in the 5’ proximal promoter. These findings suggest that maintenance of the endogenous lamin A gene in a transcriptionally silent state in PCC-4 cells is unlikely to be due to lack of binding of a specific activator or due to binding of a repressor to the proximal promoter, but may be attributed to an inactive, closed chromatin conformation. However, the possibility that binding of repressors to regulatory regions other than the 2.2 kb promoter segment might be involved in the silencing of the lamin A gene cannot be ruled out. A likely interpretation of the
Fig. 7.2. Putative model for the mechanism of transcriptional regulation of the lamin A gene. (A) Lamin A gene compacted and packaged into higher order chromatin. (B) Assembly of the PIC and activation of transcription. (C) Blockage of PIC assembly and repression of transcription. The pre-initiation complex is denoted by PIC, Sp1 molecules by S, c-Fos by F, c-Jun by J, enhancer by E and putative repressors by R1, R2, R3 and R4. The transcription initiation site is indicated by +1 and the ? indicates other cofactor molecules.
gene regulation are underway and regulatory regions of several IF genes have been characterised so far. The promoters of the constitutively expressed mouse and human lamin B1 genes, in contrast to the lamin A promoter, do not contain a TATA box. This feature is common to several housekeeping genes which, like the lamin B1 gene, are not subject to extensive regulation. The lamin B1 promoters are GC-rich and contain several consensus motifs for binding of the Sp1 transcription factor. Promoters of other IF genes which show regulated expression such as the vimentin gene have a proximal promoter element which contains a TATA box, a CAAT box, as well as several GC boxes. In addition to these sites, a distal AP-1 enhancer element has been demonstrated to be instrumental in the cell-type and growth regulation-dependent expression of the vimentin gene. In the K18 keratin gene, which carries multiple GC boxes in the 5' upstream promoter region, certain functionally important transcription factor binding sites including the AP-1 motif were found to be located in the large first intron (Oshima et al., 1990). Similarly, the K8 gene has been shown to contain important control elements in the body of the gene (Casanova et al., 1993) and several other IF genes have been found to carry both positive and negative cis-regulatory elements in their first introns, which are involved in cell-type and tissue-specific expression of these genes. Hence, it is probable that the lamin A gene might carry additional cis-regulatory enhancer or silencer elements in the more distal 5' and 3' regions, or in its long first intron.

In conclusion, these studies have provided detailed insights into the mechanisms of regulation of the rat lamin A gene. In light of the results obtained from this work, it would be of interest to examine the effects of mutagenesis of the GT and TCC motifs on promoter activity. Furthermore, studies aimed at understanding the developmental regulation of this gene should be focussed on analysis of distal regions of the promoter in a cellular chromatin context in a transgenic animal model or a stably transfected cell line.