CHAPTER VI

DISCUSSION
The role of the nuclear envelope in the regulation of exchange of macromolecules between the cytoplasm and the nucleus has been well documented. Nuclear proteins are selectively imported into the nucleus, whereas RNAs and ribosomal subunits are selectively exported from the nucleus. However, the exact mechanism of transport is not clearly understood. In the present study, important insights have been gained into the interactions of protein NLS sequences with proteins of the nuclear pore complex. The results are discussed in the light of the present status of the field, as described below.

6.1. PREPARATION AND CHARACTERIZATION OF NUCLEAR ENVELOPES

In order to understand the mechanisms involved in the regulated and highly selective exchange of macromolecules between the nucleus and the cytosol, it is first essential to characterize the nuclear envelope. One of the important features of this work has been the isolation of high quality nuclear envelopes from purified nuclei. Numerous procedures have been used in the literature for the purification of nuclear envelopes, depending on the cell type and the objective of the study. However, each method varies considerably with respect to the integrity of the outer nuclear membrane and contamination by intranuclear or cellular fractions. Since the objective in this study was to obtain envelopes with both inner and outer membranes intact, and minimal intranuclear contamination, three procedures were tried, of which the method of Kaufmann et al. (1983), was found to be most satisfactory. The original procedure has been modified in order to improve the yield and quality of the envelopes. In this method, the absence of detergent ensures the integrity of the outer membrane and the presence of DTT maintains proteins in a reduced state and prevents their cross-linking during the isolation procedure. Preparations of nuclear envelopes were analysed in detail by morphological and biochemical criteria. Morphologically, the nuclear envelopes were seen as empty vesicles of the
same size as nuclei, with both the outer and inner membranes well-preserved, and devoid of any cytoplasmic contamination. Protein/phospholipid ratio of the membranes was found to be similar to that reported by other workers (Kasper 1974, Kaufmann et al. 1983). As there is no unique marker enzyme for the nuclear envelope whose high specific activity indicates a pure envelope preparation, the biochemical purity of the envelopes was indicated by the absence of marker enzymes for mitochondria, plasma membranes and cytosol. The absence of intranuclear contamination was indicated by the low values of DNA and RNA content and by the absence of histones (the major nuclear proteins) in SDS-polyacrylamide gels.

Most studies on the protein composition of nuclear envelopes as visualized by polyacrylamide gel analysis, have identified the lamins (60-68 kDa) and a set of proteins of molecular mass 45-55 kDa as the major constituents of the envelopes. Other studies using monoclonal antibodies raised against intact envelope preparations have identified several additional proteins of the envelope and the nuclear pore complexes (Davis and Blobel 1986, Gerace et al. 1982, Snow et al. 1987). In order to identify different proteins of the nuclear envelope, which may be present in low amounts, by a rapid and convenient method, the sensitive method of radiolabelling the nuclear proteins in a surface-specific manner was carried out, using the water-insoluble, immobilized iodinating reagent "Iodogen". Iodination of intact cells by this reagent has been shown to be specific for plasma membrane proteins (Fraker and Speck 1978, Markwell and Fox 1983). Purified nuclei were radioiodinated with "Iodogen". The results obtained clearly demonstrate that only nuclear envelope proteins were labelled, as shown by (1) absence of labelling of the most abundant intranuclear proteins, the histones, in intact nuclei and their efficient labelling in lysed nuclei, and (2) absence of labelling of proteins released in the supernatants after salt extraction.
of digested nuclei to give nuclear envelopes. Thirteen distinct polypeptides of molecular masses 145, 115, 98, 85, 78, 70, 65, 54, 50, 45, 40, 38 and 36 kDa, were labelled on iodination of intact nuclei. Using biochemical fractionation methods, the 54 kDa protein was localized to the outer nuclear membrane, whereas all other polypeptides appeared to be pore-complex or integral membrane proteins. Four polypeptides (145, 98, 68 and 65 kDa) were likely to be components of the pore complex, since proteins of a similar size have been identified as pore-complex components in other studies (Snow et al. 1987). Polypeptides of molecular masses 115, 75, 70, 38 and 36 kDa did not correspond to previously identified envelope proteins and may be integral membrane proteins or hitherto unidentified pore-complex proteins. In a previous report on the iodination of nuclei by immobilized lactoperoxidase (Richardson and Maddy 1980), a similar pattern of labelling has been obtained. However, there was significant labelling (~ 30%) of histones, suggesting damage to the nuclei or nonspecific labelling of nuclear contents.

6.2. ORGANIZATION OF THE NUCLEAR LAMINA

The nuclear lamina is a ubiquitous component of the nuclear envelope, closely associated with the nucleoplasmic surface of the inner nuclear membrane. It serves as a nucleoskeleton, which is important for nuclear envelope integrity and interphase chromatin organization (reviewed in Gerace and Burke 1988). In mammalian somatic cells, the lamina is composed of three proteins called lamins A, B and C. The lamins share considerable sequence homology with the large family of intermediate filaments (McKeon et al. 1986, Fisher et al. 1986). During mitosis, when the nuclear envelope breaks down, lamins A and C are present in the soluble form in the cytosol, whereas lamin B remains associated with vesicles (Gerace and Blobel 1980, Burke and Gerace 1986).
Recent work with both *in vivo* and *in vitro* systems has shown that the disassembly of the lamina during prophase coincides with the hyperphosphorylation of the lamins (Gerace and Blobel 1980, Peter *et al.* 1990, Heald and McKeon 1991, Ward and Kirschner 1990). Phosphorylation of lamins A and C in mammalian cells and B-type lamins in avian cells has been shown to be mediated by a cell cycle-regulated kinase (Peter *et al.* 1990, Heald and McKeon 1990, Ward and Kirschner 1990) and cell cycle-regulated phosphorylation sites have been identified. An M-phase specific kinase, *cdc 2* kinase, has been shown to be directly involved in phosphorylation of lamins in avian cells (Peter *et al.* 1990).

The expression of the different lamins appears to be developmentally controlled. Using immunological techniques, the stage-specific expression of lamin A, B1, and B2 during chicken development, and lamins L1, LII, LIII, LV and LV during amphibian development have been established (Gerace and Burke 1988, Lehner *et al.* 1987). In mammals, only lamin B has been detected in embryonal stem cells and sections of early embryonic tissues; lamins A and C are present in later stages (Stewart and Burke 1987, Rober *et al.* 1989). The details of the organization of the three polypeptides, lamins A, B and C, to form the lamina are not clearly understood. Lamin B is implicated in anchoring the lamina to the nuclear membrane as it is strongly associated with the membrane. Recently, in avian and yeast cells, an integral membrane protein has been identified as a lamin B receptor, through which lamin B is associated with the inner nuclear membrane, (Worman *et al.* 1988, Worman *et al.* 1990). Binding of lamin B to the lamin B receptor has been shown to be dependent on phosphorylation of the receptor (Appelbaum *et al.* 1990).

In order to study the expression of the lamins and the nature of the lamina organization in fetal and adult mouse liver, antibodies were raised to purified lamin B and lamins A/C in rabbits. These antibodies were characterized by
immunoblotting and found to be highly specific. Using these antibodies, lamin expression was studied at different stages of liver development. The results clearly indicated that in 15 day and 19 day-old fetal liver, only lamin B is expressed, and lamins A and C are detectable only in adult liver. Since the lamina in adult cells is composed of a heteropolymer of the three lamins, but only lamin B is present in fetal tissues, the interaction of lamin B with the other lamins and membrane proteins in adult liver and in fetal liver were compared. Binding assays with purified $^{125}$I lamin B were carried out as described by Worman et al. (1988). It was observed that lamin B specifically binds to lamin A and C, and proteins of molecular mass 96, 50, 54 and 36 kDa in adult nuclear envelopes. In fetal liver nuclear envelopes, labelled lamin B binds to lamin B itself and weakly to proteins of 50-54 kDa and 34 kDa, but not to the 96 kDa protein. Binding of lamin B to lamins A and C, and the 96 kDa and 58 kDa membrane proteins in adult nuclear envelopes has been observed in other species (Worman et al. 1988, Georgatos et al. 1989).

The binding of labelled lamin B to lamin B of fetal nuclear envelopes, as seen in these studies, is unusual, and indicates some kind of modification in fetal lamin B. Although lamin B does not associate with itself normally, in vitro phospho-horylation of lamin B has been shown to significantly enhance its binding to nonphosphorylated lamin B, whereas such effects are not observed with lamin A or C (Georgatos et al. 1988). In order to check whether the hyperphosphorylation of lamin B in fetal liver envelopes may be contributing to its association with labelled lamin B, the envelopes were treated with phosphatases, and the blot binding assay was carried out with labelled lamin B. As shown in the results, it was observed that binding of labelled lamin B to lamin B was reduced considerably in phosphatase-treated fetal envelopes, whereas there was no effect on the binding of lamin A and C to labelled lamin B in phosphatase-treated adult nucle-
ar envelopes. These results suggest that lamin B is hyperphosphorylated in fetal liver cells (possibly due to rapid cell division), and this property allows homotypic association of lamin B in the fetal nuclear lamina, where lamins A and C are absent. Phosphatase-treatment of adult nuclear envelopes also reduces the binding of lamin B to the 50-54 kDa membrane proteins, which suggests that phosphorylation of the 50-54 kDa proteins is required for their interaction with lamin B. Similar effects have been reported recently for the lamin B receptor in avian erythrocytes (Appelbaum et al. 1990). Thus, the 50-54 kDa membrane proteins may be analogous to the lamin B receptor of avian cells. These results indicate that there is considerable difference in the nuclear lamina organization of fetal and adult tissues. The regulation of expression of lamins A and C during development can be studied further at the level of transcription of the lamin genes.

6.3. IDENTIFICATION AND CHARACTERIZATION OF NUCLEAR LOCATION SIGNAL-BINDING PROTEINS IN NUCLEAR ENVELOPES

The transport of a nuclear protein from the cytoplasm into the nucleus is a highly selective process which requires energy and the presence of a basic NLS in the protein. The NLS sequences for many nuclear proteins have been well characterized, especially the prototype NLS of SV40 large T antigen (Pro-Lys-Lys-Lys-Arg-Lys-Val), which has been shown to mediate the transport of nonnuclear proteins into the nucleus, when linked to them by chemical cross-linking or recombinant DNA techniques. However, the exact role of the NLS in protein transport is not clearly understood. Recent work has shown that protein import into the nucleus is a two-step process: binding of the karyophilic protein to the nuclear envelope followed by the energy-dependent translocation of the protein into the nucleus, through the pore-complex (Newmayer and Forbes 1989, Rich-
ardson et al. 1989). It is quite likely that specific proteins on the nuclear envelope may interact with the NLS, thus assisting in selective nuclear import of proteins. Several studies have been directed towards the identification of specific proteins interacting with the NLS, using either chemical cross-linking methods (Adam et al. 1989, Pandey and Parnaik, 1991) or indirect procedures (Yamasaki et al. 1989, Benditt et al. 1969, Silver et al. 1989, Yonedo et al. 1988). These are discussed in detail below.

In this work, nuclear envelope proteins that specifically recognize the prototype NLS, the SV40 large T antigen NLS, have been identified. Since short NLS peptides have been shown to target proteins to the nucleus, the strategy employed was to synthesize a peptide corresponding to the NLS for SV40 large T antigen, and to attach at the N-terminal an iodinatable photolabel (NHA-ASA) which produces a short-lived nitrene radical (Kaderbhai et al. 1988) to facilitate specific cross-linking to the binding proteins. The iodinated photolabelled NLS-peptide was photoactivated in the presence of mouse liver nuclei, nuclear envelopes and postmitochondrial supernatant and samples were analysed by SDS-PAGE in order to locate the proteins covalently linked to the labelled peptide. NLS-binding proteins were identified by demonstrating specific, saturable binding to a native NLS, which could not be competed out by a mutant NLS or unrelated peptides.

A protein of 60 kDa was shown to bind specifically to the NLS upon photoactivation with nuclei, and four proteins of molecular masses 67, 60, 53 and 47 kDa of nuclear envelopes were found to bind specifically to NLS. In order to establish that binding of NLS to nuclear envelopes is functionally significant and not an artifact, it was demonstrated that: (1) when photoactivated nuclei are processed to obtain nuclear envelopes, the 60 kDa protein is retained on the nuclear envelopes and, in addition, three more proteins of molecular masses 67,
53 and 47 kDa are seen. (2) The 60 kDa, NLS-binding protein detected in the nuclei is identical to the 60 kDa nuclear envelope protein, by two-dimensional gel analysis.

The kinetics of binding of the NLS to nuclear and nuclear envelope proteins have been studied. The binding shows saturation kinetics ($K_d$ range 8 - 40 $\mu$M for different proteins), which strongly indicates specific binding. The binding constant for the 60 kDa nuclear protein ($K_d = 8 \mu$M) is in the same range as that obtained for the kinetics of transport of NLS-BSA conjugates, although the dissociation constant obtained for a 34-residue large T antigen NLS has been reported to be still lower (approximately 0.1 $\mu$M). Since a seven amino acid long NLS is able to mediate the specific nuclear localization of BSA when conjugated to it, the nonapeptide used here should be sufficient in size for specific binding to the proteins which may be required for selective nuclear transport.

The NLS-binding proteins have been localized by biochemical fractionation of envelopes. The 53 and 47 kDa proteins appear to be peripherally bound to the outer membrane whereas the 60 and 67 kDa proteins are most probably part of the pore complex. Although the nuclear lamins also have molecular masses in the range of 60-70 kDa, it was clearly shown that the 60 and 67 kDa NLS-binding proteins are not lamins, as they can be solubilized from the envelopes with low concentration of salt or urea, unlike the lamins, and their $pI$ values are different from those of lamins (Kaufmann et al. 1983). It was also observed that a polyclonal anti-lamin B antibody does not affect the binding of the NLS to these proteins (see Section 5.3). Although solubilized NLS-binding proteins retain the ability to bind to the native NLS, their affinity for the native NLS is lower when compared to that in intact nuclear envelopes. This suggests that the location of NLS-binding proteins in the nuclear envelope is an important
feature for specific signal recognition. Competition data from other studies also suggest that the solubilized receptors have lesser affinity for the T antigen NLS (Benditt et al. 1989). Using an indirect approach, by raising antibodies to a putative receptor sequence (anti-DDDED), Yoneda and co-workers (1988) have identified NLS-binding proteins of molecular masses 59 and 69 kDa in nuclear pores and 65, 54 and 50 kDa in nuclear envelope extracts. The results from this work are consistent with these findings.

Using a similar approach, Adam and co-workers (1989) have used a 34-residue long T-antigen signal sequence and the cross-linking reagent, disuccinimidyl suberate, and have identified a high affinity 60 kDa NLS-binding protein in the nucleus, which may also be present in the cytosol. These authors have argued for a role of the cytosolic protein in nuclear transport. Although a protein of similar size has been identified in nuclei and nuclear envelopes in this study, the absence of binding of the native NLS to postmitochondrial supernatant proteins indicate that there are no proteins in this fraction which bind specifically to the NLS. The cytosolic 60 kDa protein identified by Adam et al. (1989) may have weak affinity for the NLS. Yoneda et al. (1988) were also unable to detect any cytosolic binding proteins by immunoprecipitation with anti-DDDED antibodies. In an in vitro nuclear transport system using proteins translated from SP6 plasmid-generated RNAs, described by Parnaik and Kennady (1990), there does not seem to be a requirement of cytosolic factors for nuclear transport. In in vitro systems with purified nuclear proteins, a requirement for cytosolic factors has been shown in the case of nucleoplasmin only and not for SV40 large T antigen and other nuclear proteins (Benditt et al. 1989, Markland et al. 1987). Cytosolic proteins which bind nonspecifically to the native NLS of T antigen and to a modified NLS with poor transport function have also been described, but their role in nuclear transport is unclear (Yamasaki et al. 1989, Lanford et al. 1988).
Although ATP is required for the translocation of proteins across the nuclear pores, it is not required for the initial binding of karyophilic proteins to the pore complex (which can occur at 4°C and in absence of ATP), (Newmayer and Forbes 1988, Richardson et al. 1988). In these studies, addition of ATP did not enhance the binding of NLS to the nuclear or nuclear envelope proteins, as observed by other investigators also (Adam et al. 1989, Benditt et al. 1989). Similarly, wheat germ agglutinin, which has been shown to bind certain nuclear envelope proteins (Snow et al. 1987, Newmayer et al. 1986), but not to the same site to which SV40 T antigen NLS binds (Newmayer and Forbes, 1988), did not affect the binding of NLS peptides to envelope proteins in this study.

The results from this work are compatible with a model recently proposed for protein import into the nucleus (Akey 1990). According to this model, protein transport into the nucleus takes place in three steps; (a) binding of NLS containing proteins at the periphery of the pores, which results in rapid perinuclear accumulation of nuclear proteins, (b) binding of the nuclear protein to the central transporter region, and (c) dilation of the pore to permit translocation of the protein into the nucleus. The peripherally located NLS-binding proteins of molecular masses 53 and 47 kDa may be involved in the first step of nuclear protein import, in which binding of the nuclear protein occurs at the periphery of the pore. The 60 kDa and 67 kDa NLS-binding proteins may be part of the central transporter assembly (the fractionation studies described in Section 4.3.4 suggest that these proteins are part of the pore complex).

6.4. FUNCTIONAL CHARACTERIZATION OF NUCLEAR PORE PROTEINS USING ANTIBODIES

From the studies described above, it is evident that a number of proteins of the nuclear envelope are involved in the import of karyophilic proteins. In order
to understand the role of these proteins in transport, it would be useful to have immunological probes specific for different proteins. Until recently, only two proteins of the pore complex had been identified and well-characterized using antibody probes: a membrane glycoprotein, gp190, and a pore complex glycoprotein of 62 kDa (Gerace et al. 1982, Davis and Blobel, 1986). In the last few years, two groups of workers have obtained monoclonal antibodies that recognize different proteins of the nuclear envelope. A subset of these proteins has been shown to be recognized by monoclonal antibodies which specifically interact with glycosyl chains containing GlcNAc moieties linked to serine or threonine residues (Snow et al. 1987, Park et al. 1987, Holt et al. 1987, Hanover et al. 1987, Davis and Blobel 1986). These antigens containing GlcNAc residues have been termed "nucleoporins' and have been localized to the nuclear pores by immunoelectron microscopy of rat liver nuclei (Davis and Blobel 1986) or by immunoblotting of nuclear envelope fractions (Snow et al. 1987).

In the studies described here, monoclonal antibodies to proteins of rat nuclear envelopes have been obtained and characterized in detail by Western blot analysis, immunofluorescence and NLS-binding assays. Two antibodies potentially useful for understanding the interaction of the NLS sequence with its nuclear pore receptors were obtained: E2 and its derivatives, and A8. The monoclonal antibodies E2B11, E2B9, E2B10 and E2E3 recognized a common set of nuclear envelope proteins of molecular masses 76, 67-60 and 42 kDa, suggesting that these polypeptides share a common epitope. The possibility that this epitope may be GlcNAc, as reported for the nucleoporins in earlier studies, was checked by carrying out the following experiments. Monoclonal antibodies were preincubated with the free sugar GlcNAc before Western blot analysis or blots were preincubated with WGA before addition of antibodies. In each case, binding of the antibodies to proteins of the nuclear envelope would have been
inhibited if GlcNAc was the common epitope on these proteins. However, there was clearly no inhibition of antibody-antigen binding on Western blots. These results indicate that the shared epitope on the polypeptides recognized by the E2B11, E2B9, E2B10 and E2E3 monoclonal antibodies is not GlcNAc. The E2B11 antibody was shown to inhibit the binding of the NLS sequence to its receptors by photoaffinity assays. Since this antibody recognizes proteins in the range of 60-70 kDa on Western blots, it may directly bind to the 60 and 67 kDa NLS-binding proteins and may interfere indirectly with binding of the NLS peptide to the 53 and 47 kDa NLS-binding proteins. Furthermore, the proteins recognized by E2B11 have been localized to the nuclear pores by immunofluorescence assays. These results strongly suggest that NLS recognition takes place at the nuclear pore complex.

The A8 antibody recognises a 53 kDa protein on Western blot analysis of nuclear envelopes. This antibody increased the affinity of the NLS for the 53 kDa NLS-binding protein by about four-fold in photoaffinity assays, possibly by causing a conformational change in the 53 kDa protein. Some of the other antibodies which specifically stained the nuclear envelope (A2, and H6) did not affect NLS-binding in photoaffinity assays. The effect of these antibodies and others on in vitro transport of nuclear proteins will be determined in further studies.

There have been relatively few studies in the recent literature on the functional characterization of nuclear transport of macromolecules using antibodies. One of the anti-nucleoporin antibodies has been shown to block the efflux of 5S RNA as well as the import of nucleoplasmin (Featherstone et al. 1989). Recently, Akey and Goldfarb (1989) have used WGA and anti-nucleoporin antibodies to localize the nucleoporins to the central region of the pore, within a discrete structure which they have termed the "transporter". Some of the monoclonal antibodies raised against mammalian nuclear envelope proteins have been found to
cross-react with yeast nuclear envelope proteins (Aris and Blobel 1989, Davis and Fink 1990, Nehrbass et al. 1990). Using these antibodies, two yeast nuclear envelope proteins encoded by the genes NSP1 and NUP1 have been identified as pore complex constituents and are essential for cell viability. These two proteins have similar heptad repeat sequences within a central domain, a motif that may constitute the cross-reactive epitope recognized by certain monoclonal antibodies, and may represent a general feature of the nucleoporins in addition to the presence of O-linked GlcNAc residues (Davis and Fink 1990, Nehrbass et al. 1990. The E2B11 antibodies characterized in this work may also recognize a similar heptad repeat motif. This is yet to be established.

6.5 CONCLUSIONS AND FUTURE PROSPECTS

The significant findings in this study are the identification and characterization of NLS-binding proteins at the nuclear pores which specifically recognize the prototype NLS of SV40 large T antigen. An important contribution of this work has been the production of monoclonal antibodies to proteins of the nuclear envelope, which have considerable potential for use in functional assays for nuclear transport.

The major advances in this field have proceeded along two directions. Firstly, it is now established that nuclear proteins enter the nucleus in a signal sequence-dependent and energy-dependent process. Secondly, it is becoming evident that this basic mechanism can be modulated in different ways. Recent studies suggest that regulation of macromolecular transport across the nuclear envelope is an important step in gene regulation. Some regulatory nuclear proteins such as transcription factors and proto-oncogene encoded protein kinases remain anchored in the cytoplasm, and enter the nucleus only in response to specific signals. This suggests that signal transduction from the cytoplasm into
the nucleus may involve the regulation of compartmentalization of transducing molecules. Catalytic sub units of protein kinase A translocate to the nucleus in response to elevated concentrations of cAMP, but remain otherwise in the cytoplasm, associated with the regulatory subunits (Nigg 1989, Nigg 1990). Similarly, the immunoglobulin transcription factor NF-kB remains in the cytoplasm of nonexpressing B-cells by forming a complex with an inhibitory protein, I-kB. However, when the cells are treated with various stimulators of protein kinase C, this enzyme phosphorylates I-kB which in turn results in dissociation of the I-kB/NF-kB complex and translocation of free NF-kB into the nucleus (Ghosh and Baltimore 1990, Shirakawa and Mizel 1989). Recently a gradient of nuclear localization of the dorsal protein (a product of the dorsal gene, which is part of a complementation group required for the determination of dorsoventral polarity of Drosophila at the syncytial blastoderm stage, has been shown to be responsible for establishing the gradient of dorsoventral positional information (Roth et al. 1990, Rushlow et al. 1990, Steward 1990). Further studies on regulated transport of different proteins should give new insights into the different ways in which nuclear transport and gene activity can be modulated. Certain important questions on the biochemical nature of the pore still remain to be answered. How does the pore orifice expand to allow the translocation of large proteins? Are there different receptors for recognition of other classes of signals? Some of these questions can be best approached by using specific monoclonal antibodies in functional assays.