THE ROLE OF NUCLEOPHOSMIN FUSION SEQUENCES IN THE ONCOGENIC ACTIVATION OF THE (2;5) TRANSLOCATION PROTEIN, NUCLEOPHOSMIN-ANAPLASTIC LYMPHOMA KINASE (NPM-ALK)

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa, in fulfillment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 1996

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other university. The work described here was performed in the laboratory of Dr. Stephan W. Morris at St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

Daniela Bischof

18th day of December, 1996.

ANIMAL ETHICS CLEARANCE

Protocols involving the use of animals were approved by the Animal Resources Committee at St. Jude Children's Research Hospital, Memphis, Tennessee, USA, animal protocol number 206.

ABSTRACT

The *NPM-ALK* fusion gene, formed by the t(2;5)(p23;q35) in non-Hodgkin's lymphoma encodes a 75kDa hybrid protein that contains the amino-terminal 118 amino acid residues of the nucleolar phosphoprotein nucleophosmin (NPM) joined to the entire cytoplasmic portion of the receptor tyrosine kinase, anaplastic lymphoma kinase (ALK). The transforming ability of NPM-ALK is demonstrated and it is shown that oncogenesis by the chimaeric protein requires the activation of its kinase function as a result of oligomerisation mediated by the NPM segment. NPM-ALK was shown to homo-oligomerise *in vivo* in addition to hetero-oligomerising with normal NPM. Immunostaining experiments of the t(2;5)-containing lymphoma cell line SUP-M2 revealed NPM-ALK to be localized within both the cytoplasmic and nuclear/nucleolar compartments. An intact NPM segment is absolutely required for NPM-ALK-mediated oncogenesis, as indicated by the observation that three different NPM-ALK mutant proteins lacking non-overlapping portions of the NPM segment each failed to form complexes, lacked kinase activity *in vivo*, and failed to transform cells. However, NPM could be replaced in the fusion protein with the portion of the unrelated translocated promotor region (TPR) protein that activates the TPR-MET fusion kinase by mediating dimerisation through its leucine zipper motif. This engineered TPR-ALK hybrid protein, which transformed cells with essentially

equivalent potency to NPM-ALK, was localised within the cytoplasm of cells. These data indicate that the nuclear/nucleolar localisation of NPM-ALK, which probably occurs because of transport via shuttling activity of NPM, is not required for oncogenesis. Further, the activation of the truncated ALK protein by a comp' 'ely heterologous oligomerisation domain suggests that the functionally important role of the NPM segment of NPM-ALK in transformation is restricted to the formation of kinase-active oligomers and does not involve the alteration of normal NPM function.

ACKNOWLEDGEMENTS

To Professor Kate Hammond for her support.

To Dr. Stephan Morris for his support, encouragement and patience.

PUBLICATIONS EMANATING FROM THIS STUDY

Bischof, D. and Morris, S.W. Role of the NPM portion of the non-Hodgkin's lymphoma-associated NPM-ALK fusion protein in oncogenesis. Mol Cell Biol (In Press).

Mason, D.Y., Pulford, K.A.F., Bischof, D., Lamant, L., Delsol, G. and Morris, S. Nucleolar localisation of the nucleophosmin-ALK tyrosine kinase not required for malignancy. (In Preparation).

ABBREVIATIONS

AC	acidic region
ALCL	anaplastic large cell lymphoma
ALK	anaplastic lymphoma kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BBS	BES-buffered saline
bp	basepair(s)
BCR	breakpoint cluster region
BES	N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid
°C	degrees Celsius
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CCOH	carboxy terminus
CSF-1R	colony-stimulating factor 1 receptor
DAG	diacylglycerol
DAPI	4', 6-diamidine-2'-phenylindole dihydrochloride
ddATP	dideoxy adenosine triphosphate
ddCTP	dideoxy cytidine triphosphate

ddGTP	dideoxy guanosine triphosphate
ddTTP	dideoxy thymidine triphosphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EGFR	epidermal growth factor receptor
FCS	foetal calf serum
FeSV	feline sarcoma virus
Fr3T3	Fisher rat 3T3
GAP	GTPase activating protein
GRB-2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HCl	hydrochloric acid
HGFR	hepatocyte growth factor receptor
HGF/SF	hepatocyte growth factor/scatter factor
HIV	human immunodeficiency virus
IGF-1R	insulin-like growth factor 1 receptor
Inv	inversion
IP_3	inositol triphosphate
IR	insulin receptor

IRS-1	Insulin receptor substrate 1
JM	juxtamembrane domain
kb	kilobase(s)
kDa	kilo Dalton(s)
LB	Luria broth
LC NHL	large cell non-Hodgkin's lymphoma
LTK	leukocyte tyrosine kinase
LZ	leucine zipper
MAPK	mitogen-activated protein kinase
MAPKAP	MAPK-activated protein
MB	metal binding domain
$MgCl_2$	magnesium chloride
MEK	MAPK/ERK kinase
MLF1	myelodysplasia/myeloid leukaemia factor 1
$MnCl_2$	manganese chloride
mRNA	messenger RNA
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate
NaOH	sodium hydroxide
NGF	nerve growth factor

NH2	amino terminus
NLS	nuclear localisation signal
NP-40	Nonidet P-40
NPM	nucleophosmin
P	phosphate group
pBS	pBluescript SK(+)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PI3'-kinase	phosphatidylinositol kinase
Pipes	piperazine-N,N'-bis(2-ethanosulfonic acid)
PKC	protein kinase C
PLCγ	phospholipase C gamma
PLZF	promyelocytic leukaemia zinc finger
PML	promyelocytic leukaemia
PTB	phosphotyrosine-binding
PTP1D	protein tyrosine phosphatase 1D
PVDF	polyvinylidene difluoride
RARα	retinoic acid receptor α

rATP	riboadenosine triphosphate
RIα	cyclic AMP-dependent protein kinase
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacylamide gel electrophoresis
SH2	SRC homology 2
SH3	SRC homology 3
SHC	SRC homology and collagen
SOS	Son-of-sevenless
SP	signal peptide
t	translocation
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TFG	TRK fused gene
TM	transmembrane domain
TPM3	non-muscle tropomyosin
TPR	translocated promotor region
Tris	Tris(hydroxymethyl)aminomethane
TRK	tropomyosin receptor kinase
UT	untranslated

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1. INTRODUCTION

1.1 Signalling by receptor tyrosine kinases

Protein tyrosine kinases are present in all multicellular organisms where they play crucial roles in signal transduction. One class of these enzymes, designated intracellular kinases includes members of the SRC family of kinases which are associated with inner parts of the cell membrane, as well as members of the ABL kinase family, which encode intracellular proteins with the ability to localise to the nucleus (Rodrigues and Park, 1994). The second class, tyrosine kinase receptors, include the epidermal growth factor receptor (EGFR), insulin receptor (IR), insulin-like growth factor 1 receptor (IGF-1R), platelet-derived growth factor receptors (PDGFR), fibroblast growth factor receptors (FGFR), hepatocyte growth factor receptor (HGFR/MET), and the tropomyosin receptor kinase (TRK) family. These members, all of which possess single transmembrane domains separating the extracellular domains from the intracellular domains, typically contain one or several copies of immunoglobulin-like regions, cysteine-rich domains, or other domains (reviewed by Fantl et al., 1993).

Receptor tyrosine kinases are activated after either homodimerisation or heterodimerisation. The different isoforms of platelet-derived growth factor

(PDGF), for example, can induce the formation of different dimeric forms of its receptors: whereas PDGF-AA induces only PDGFRαα homodimers, PDGF-AB induces both PDGFRαα homodimers and PDGFRαβ heterodimers, and PDGF-BB induces all three forms of receptors. EGFR can homodimerise with itself or form heterodimers with ERBB2, a receptor tyrosine kinase with extensive homology to EGFR (Coussens et al., 1985; Yamamoto et al., 1986). The insulin and insulin-like growth factor 1 receptor family represent a marked exception to the above mechanism. These receptors exist in the cell membrane as disulfide-bonded homodimers and heterodimers of receptor subunits. This means that ligand binding does not induce receptor dimerisation, but presumably causes a conformational change in the existing dimeric structure, leading to receptor activation. In addition, autophosphorylated tyrosine residues in the receptor are not essential for binding of downstream components in the signal transduction pathways. Instead, the receptor phosphorylates insulin receptor substrate 1 (IRS-1), which mediates subsequent interactions with proteins containing SRC homology 2 (SH2) domains (reviewed by Heldin, 1995).

Receptor dimerisation is followed by receptor autophosphorylation, which principally occurs by one receptor molecule phosphorylating the other in the dimer. This autophosphorylation occurs on two different classes of tyrosine

residues. On the one hand, autophosphorylation is typically seen on a conserved tyrosine residue within the kinase domain(s). In the cases of the insulin receptor and MET, phosphorylation of tyrosine residue at this and neighbouring sites leads not only to an increase in the kinase activity but also to phosphorylation of other sites in the receptor or its substrates. The initialising event in autophosphorylation is presently unknown. One possibility is that the monomeric receptor has a low basal kinase activity, sufficient to phosphorylate and activate the companion receptor after dimerisation. This reaction would then be followed rapidly by reciprocal phosphorylation. Alternatively, interactions between intracellular domains of the dimerised receptors may induce conformational changes that promote increased kinase activity. It should be emphasised that not all receptors are regulated by phosphorylation in the kinase domain; for example, the conserved tyrosine residue in the kinase domain of EGFR does not appear to be autophosphorylated (reviewed by Heldin, 1995).

The second class of autophosphorylation sites are often located outside the kinase domain(s) and serve the important function of creating docking sites for downstream signal transduction molecules containing SH2 domains. Numerous proteins involved in signal transduction are modular, having structures suggesting that they were "cobbled together from a tool kit of

functional domains", and some of these domains mediate protein-protein associations (Mayer and Baltimore, 1993). Two such domains are SH2 and SRC homology 3 (SH3) which appear to play critical roles in the formation of signalling complexes (figure 1.1). SH2 domains, consist of about 100 amino acids folded in such a way as to form a binding pocket for a phosphorylated tyrosine and the immediately surrounding amino acids. SH3 domains, consisting of approximately 50 amino acids, are proline-rich and recognise one or more proline residues. Such regions are found in a wide variety of protein contexts: in association with catalytic domains and the non-receptor protein tyrosine kinases; within structural proteins such as fodrin or tensin; and in a group of small adaptor molecules that consist almost entirely of SH2 and SH3 domains, including CRK, NCK and growth factor receptor-bound protein 2 (GRB-2) (Mayer and Baltimore, 1993; Pawson, 1995).

Although SH3 domains frequently co-exist with SH2 domains, they are also found in separate contexts. They have been identified in the products of several genes in budding and fission yeast, and have been linked to processes such as budding (BEM1 and ABP1), cell fusion (FUS1), and RAS guanine nucleotide exchange (CDC25). SH2 domains have not been identified in yeast, which also lack SRC-related tyrosine kinases. Evidence suggests

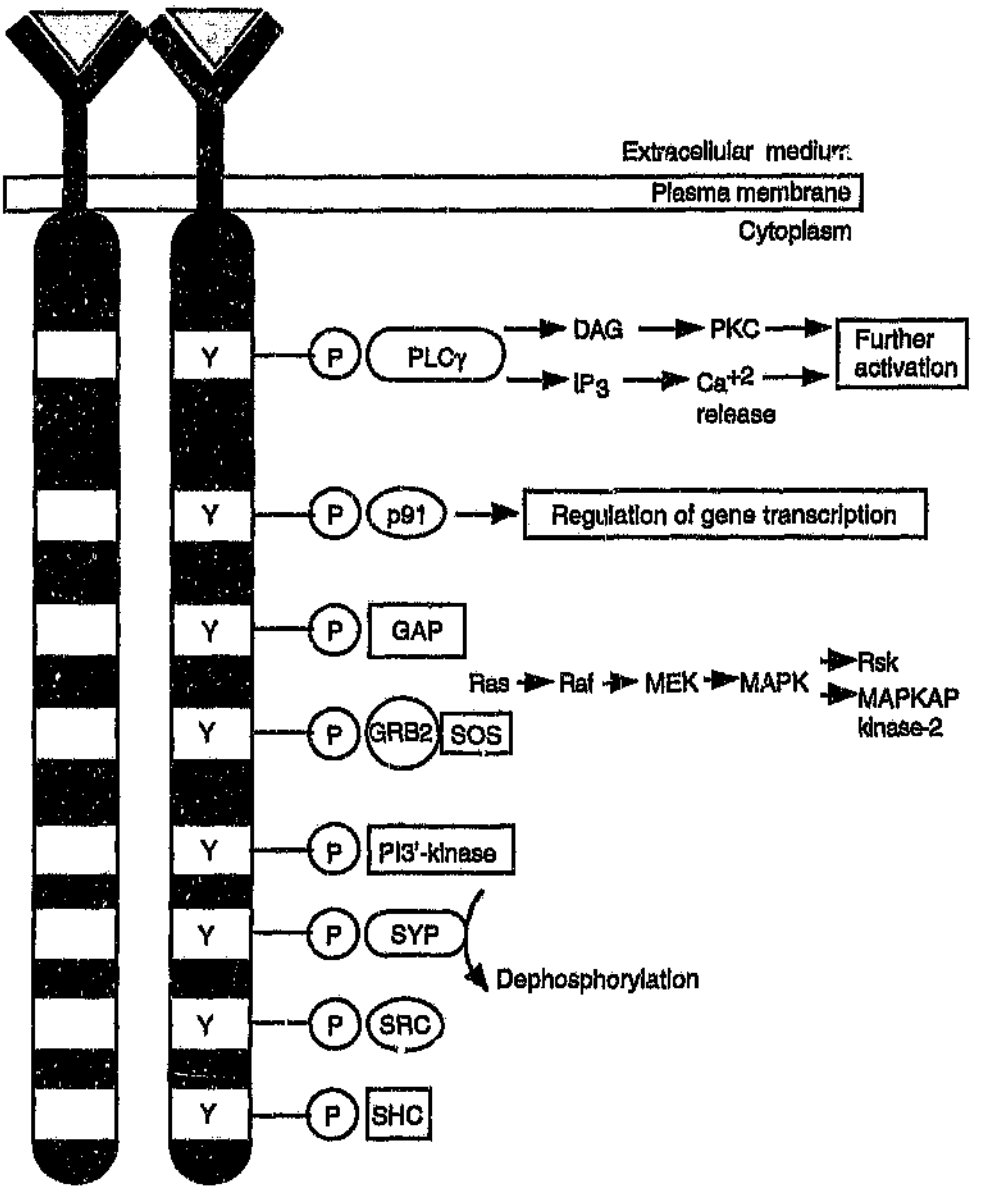


Figure 1.1. Interaction of a representative receptor tyrosine kinase with SH2/SH3 signalling molecules. After receptor activation and subsequent autophosphorylation, signal transduction molecules are recruited. Proteins containing SH2 domains associate with specific phosphotyrosines of the receptor thus providing for downstream signal transduction molecules (modified from Johnson and Vaillancourt, 1994; Pawson and Schlessinger, 1993).

that SH3 domains are involved in the control of small RAS-like guanine nucleotide-binding (G) proteins. The most compelling results relate to the cloning of the *3BP1* gene, whose product binds directly to the SH3 domains of c-ABL. This short proline-rich peptide contains a sequence distinct from the SH3-binding site that is related to a group of proteins with GTPase-activating activity toward members of the RHO/RAC family of small G proteins. Similar proline-rich sequences were identified in Son-of-sevenless (SOS) which was initially identified as a component of a signalling pathway initiated by tyrosine kinase receptors (Pawson and Gish, 1992; Pawson and Schlessinger, 1993).

Receptor autophosphorylation acts as a molecular switch to create binding sites for the SH2 domains of cytoplasmic signalling proteins, which consequently become targets for activation. SH2 domains directly recognize phosphotyrosine, but for high affinity binding to occur the phosphotyrosine must be embedded within a specific amino acid sequence. Residues immediately carboxy-terminal to the phosphorylated tyrosine, especially those at the +1 and +3 positions, appear to provide selectivity for specific SH2 domains, such as those found in SRC and LCK. As mentioned earlier, the insulin receptor employs a variation in this strategy. Its autophosphorylation stimulates receptor kinase activity, but there is no

evidence that such activation induces strong association with signalling proteins. Instead, the insulin receptor interacts with a 185kDa protein known as IRS-1, which contains many potential tyrosine phosphorylation sites that lie within consensus SH2-binding elements (Sun et al., 1991). Tyrosine phosphorylated IRS-1 is found in association with both GRB-2 and the p85 subunit of phosphatidylinositol 3'-kinase (PI3'-kinase). Thus, rather than incorporating its SH2-binding sites as an intrinsic part of the receptor, the insulin receptor relies on a discrete SH2-docking protein (Pawson, 1995; Pawson and Gish, 1992).

Another example by which targets of receptor tyrosine kinases transmit biological signals includes the SH2-containing protein p91. PDGF can induce the transcription factor SIF to bind to a regulatory element, SIE, in the c-*FOS* promotor. Protein p91, which was identified as a component of the interferon-α inducible transcription complex, ISGF-3, apparently mediates these events. Tyrosine phosphorylated p91 accumulates in the nucleus where it can bind the SIE element and promote transcription (reviewed in Kazlauskas, 1994).

In the nematode *Caenorhabditis elegans*, the *SEM*-5 gene encodes a small protein with the structure SH3-SH2-SH3 that appears to link a receptor

tyrosine to the RAS pathway (Pawson and Gish, 1992). The human GRB-2 protein is similar in structure and function to the *SEM-5* product, suggesting that this pathway has been highly conserved in evolution (Lowenstein et al., 1992; Pawson and Gish, 1992). GRB-2 binds EGFR directly through tyrosine 1068 and indirectly through the SRC homology and collagen (SHC) site. It also interacts with PDGFR directly through tyrosine 716 and indirectly through binding to a phosphorylated SYP/protein tyrosine phosphatase 1D (PTP1D) (Heldin, 1995; Schlessinger, 1994).

GRB-2 binds directly to murine SOS 1, a guanine nucleotide releasing factor for RAS GTPase, through two SH3 domains. S[illegible] is also associated with activated receptor tyrosine kinases that bind GRB-2, and with the GRB-2 binding protein SHC. Overexpression of *Drosophila* SOS stimulates morphologic transformation of rodent fibroblasts (Egan et al., 1993). GRB-2 can also bind tyrosine kinase substrates through its SH2 domain (Lowenstein et al., 1992). This bifunctionality allows GRB-2 to serve as an adaptor molecule that integrates upstream signallers (the kinases) with elements two steps further down the cascade (SOS) (Egan et al., 1993).

Signal transduction pathways become more complex when one considerc that p85 PI3'-kinase, phospholipase C gamma (PLCγ) and GTPase-activating

protein (GAP) all have two SH2 domains and can themselves be phosphorylated on tyrosine upon receptor dimerisation. The binding of tyrosine kinase receptors to SH2-containing proteins affords at least three potential signalling mechanisms: (a) alteration of subcellular localisation, (b) conformational changes, and (c) phosphorylation by the activated receptor (Mayer and Baltimore, 1993).

SH2 domains have other functions besides those already mentioned. Members of the c-SRC family are held in a catalytically inactive state by phosphorylation of a carboxy-terminal tyrosine residue. Mutation of this tyrosine to phenylalanine renders these proteins oncogenic by increasing the kinase activity of c-SRC members *in vitro* and *in vivo*. The SH2 domain of SRC has moderately high affinity for its phosphorylated carboxy-terminal peptide, and has been proposed to interact in *cis* with this site to mask the catalytic domain. On the other hand, when the protein is constitutively active, mutations in the SH2 domain generally decrease transforming activity, indicating a second, positive role for this motif. Deletion or mutation of the SH3 domain in non-receptor tyrosine kinases generally leads to oncogenic activation of the proto-oncogene kinase, suggesting that the SH3 domain binds to an inhibitor of kinase activity (Mayer and Baltimore, 1993).

SHC, a modular protein with a carboxy-terminal SH2 domain, also contains a phosphotyrosine-binding (PTB) domain on the amino terminus and a central glycine/proline-rich motif with regions of homology to the α1 chain of collagen. The PTB domain has a minimal binding region of approximately 160 amino acids and is involved in protein-protein interactions. SHC is presumably involved in RAS activation through its interaction with GRB-2 and SOS. Mouse fibroblasts that overexpress SHC become transformed in culture and form tumours in nude mice. SHC, which is essentially cytoplasmic, is highly conserved and widely expressed (Pelici et al., 1992; van der Geer and Pawson, 1995)

1.2 Oncogenic activation of receptor tyrosine kinases

Many tyrosine kinase receptors are activated by means other than ligand binding, for example, activation after the binding of antibodies, where the Fab fragments are usually inactive (Heldin, 1995). Insertion of an extra cysteine residue in the extracellular juxtamembrane region of EGFR leads to formation of a constitutively active dimeric receptor (Sorokin et al., 1994). Furthermore, mutated forms of some receptor tyrosine kinases have been identified as transforming oncogenes. Frequently, the activating mechanism

is a gene rearrangement that leads to production of a hybrid protein containing the kinase domain of the receptor fused to a novel protein sequence. The amino-terminal fusion partners are often domains of proteins that normally undergo oligomerisation. Examples include sequences from the translated promotor region (TPR) fused to MET, non-muscle tropomyosin (TPM3) fused to TRK, the regulatory subunit of the cyclic AMP-dependent protein kinase (RIα) fused to RET, nucleophosmin (NPM) sequences fused to the anaplastic lymphoma kinase (ALK), and TEL fused to PDGFRβ (Golub et al., 1994; Martin-Zanca et al., 1986; Morris et al., 1994; Park et al., 1986; Rodrigues and Park, 1994; Takahashi et al., 1985) (table 1.1).

Receptor tyrosine kinases can also become oncogenic through the acquisition of point mutations that result in constitutive activation of the catalytic domain (Yarden and Ullrich, 1988). This mechanism is exemplified by the *NEU* (*ERBB2*) oncogene product, which contains a single amino acid exchange in the transmembrane region, that promotes receptor oligomerisation, and by the colony-stimulating factor 1 receptor (CSF-1R) whose transforming activity derives from a mutation in the extracellular domain (Roussel et al., 1988; Weiner et al., 1989; Woolford et al., 1988).

Gene rearrangement	Chromosomes involved	Cancer	References
NPM-ALK	t(2;5)(p23; q35)	Non-Hodgkin's lymphoma	a
TPR-MET	t(1;7)(q25; q21-31)	Gastric carcinoma	b,c,d
TPM3-TRK	Inv(1)(q31; q23-24)	Colon & thyroid carcinoma	e,f,g,h
TPR-TRK	Inv(1)(q25; q23-24)	Thyroid carcinoma	i
TFG-TRK	t(1;3)(q23-24; q?)	Thyroid carcinoma	j
L7a-TRK	t(1;9)(q23-24; q34)	Breast carcinoma	k,l
TEL-PDGFβ	t(5;12)(q33; p13)	Myeloid leukaemia	m
D10S170-RET	Inv(10)(q11.2; q21)	Thyroid carcinoma	n
Rlα-RET	t(10;17)(q11.2;q23-24)	Thyroid carcinoma	o

Table 1.1. Oncogenic activation of some receptor tyrosine kinases. Tyrosine kinases can become constitutively activated by gene rearrangements through fusion with novel proteins that normally undergo oligomerisation. **a** (Morris et al., 1994), **b** (Park et al., 1986), **c** (Miranda et al., 1994), **d** (Soman et al., 1991), **e** (Martin-Zanca et al., 1986), **f** (Radice et al., 1991), **g** (Morris et al., 1991), **h** (Sozzi et al., 1992), **i** (Greco et al., 1992), **j** (Greco et al., 1995), **k** (Kozma et al., 1988), **l** (Yon et al., 1989), **m** (Golub et al., 1994), **n** (Ishizaka et al., 1992), **o** (Bongarzone et al., 1993).

Although the mechanism for oncogenic activation of receptor tyrosine kinases may vary, they invariably lead to constitutive activation of normally tightly regulated enzymes (Rodrigues and Park, 1994).

1.2.1 TPR-MET

The human *TPR* gene, which maps to chromosome 1, encodes two alternatively spliced transcripts - one encoding a protein of 726 amino acids and the other a protein of 2094 amino acids. Both of these ubiquitously expressed proteins have been localised to the cytoplasmic surface of the nuclear pore complex and both are predicted to have extensive regions of α-helices and several stretches of heptad repeats which is characteristic of proteins with a coiled coil conformation. TPR is weakly homologous to the α-helical domains of tropomyosin, spectrin, laminin B1, the *Drosophila* glued protein and the tail region of myosin heavy chain (Byrd et al., 1994; Chan et al., 1987; Mitchell and Cooper, 1992; Mitchell and Cooper, 1992; Park et al., 1986).

MET, the receptor for the hepatocyte growth factor/scatter factor (HGF/SF), is a heterodimer of 190kDa consisting of a 50kDa α subunit associated with a 140kDa β subunit. Multiple forms of this receptor tyrosine

kinase have been identified (Rodrigues et al., 1991; Vigna et al., 1994). MET is related to the kinase domains of the human insulin receptor, EGFR and the murine v-ABL oncoprotein (Dean et al., 1985; Reddy et al., 1983). The *MET* proto-oncogene, which is predominantly expressed in fibroblast and epithelial cell lines, maps to human chromosome 7q21-31 (Park et al., 1986). The tight genetic linkage of the *MET* locus with the hereditary genetic disease cystic fibrosis, led to the assignment of the cystic fibrosis gene to the 7q21-31 region (Dean et al., 1985; White et al., 1985).

The HGF/SF ligand and MET regulate growth, motility and morphogenesis of cells *in vitro*. In mouse embryos deficient in MET, the diaphragm and limb bud are no longer colonised by myogenic precursor cells, consequently skeletal muscles of the limbs and diaphragm do not form. These abnormalities appear to be responsible for death during embryonic development (Bladt et al., 1995). Moreover the *MET* gene was found to be amplified in cell lines derived from poorly differentiated human gastric carcinomas and in spontaneously transformed murine NIH3T3 fibroblasts (Cooper et al., 1986; Giordano et al., 1988; Giordano et al., 1989). Amplification in these cells does not appear to be accompanied by any gene rearrangement, but it is associated with MET overexpression in 60% of osteosarcomas, for example (Ferracini et al., 1995; Rodrigues et al., 1991).

Another mechanism for oncogenic activation of *MET* entails its fusion with *TPR*, as seen in human osteogenic sarcoma cells treated *in vitro* with the clastogenic carcinogen N-methyl-N'-nitronitrosoguanidine. The chimaeric protein generated by chromosomal translocation has a molecular mass of 60 to 65kDa and contains the amino-terminal sequence from TPR and the carboxy-terminal sequence from MET (Chan et al., 1987; Park et al., 1986). The extracellular and transmembrane domains and a portion of the cytoplasmic region of MET are deleted (Park et al., 1987). In the 523 amino acid TPR-MET, 142 TPR codons are followed by 381 inframe MET amino acid residues (Chan et al., 1987).

Deletion of TPR from TPR-MET abolishes the transforming ability of this protein. A leucine zipper region within the TPR sequences mediates dimerisation of TPR-MET and is thus responsible for constitutive activation of the MET kinase. As a result of gene rearrangement, TPR-MET is localised to the cytoplasm (Rodrigues and Park, 1993). In addition to activating MET, TPR is also known to activate the serine/threonine kinase RAF, and the receptor tyrosine kinase TRK (Greco et al., 1992; King et al., 1988).

1.2.2 TPM3-TRK

The TRK family of receptor tyrosine kinases, including TRK (or TRKA), TRKB and TRKC, mediate the trophic effects of the nerve growth factor (NGF) family of neurotrophins (Barbacid, 1994). TRK, the receptor for nerve growth factor is essential for the development of the peripheral and central nervous systems. Mice deficient in TRK have severe sensory and sympathetic neuropathies and most die within a month of birth (Kaplan et al., 1991; Smeyne et al., 1994).

The *TRK* proto-oncogene encodes two isoforms (790 and 796 amino acids each) that yield glycosylated proteins of 140kDa (Barker et al., 1993; Martin-Zanca et al., 1989). They differ in the presence of six amino acids located in the extracellular domain (Barbacid, 1994). The 796 residue protein is expressed mainly in neuronal cells, while the 790 amino acid isoform is found primarily in non-neuronal cells (Barker et al., 1993).

Both *TPM3* and *TRK* are located on the long arm of chromosome 1: the former at 1q31 and the latter at either 1q23-24 or 1q32-41 (Miozzo et al., 1990; Morris et al., 1991; Radice et al., 1991). Thus, the generation of the hybrid protein is due to an intrachromosomal rearrangement.

TPM3-TRK was cloned from a human colon carcinoma by DNA mediated transformation of NIH3T3 cells. The 70kDa TPM3-TRK protein consists of 641 amino acids, 221 of which are derived from TPM3 with the remaining 420 representing the TRK receptor tyrosine kinase (Martin-Zanca et al., 1986). Oncogenic activation of TRK has been attributed to the coiled coil structure of TPM3 (Barnes et al., 1989; Sodek et al., 1972). Both the TPM3 and the TRK portions are required for proper transformation (Coulier et al., 1989). Activated TRK has also been found in papillary thyroid carcinoma and breast carcinoma (Kozma et al., 1988; Sozzi et al., 1992; Ziemiecki et al., 1990).

In addition to its constitutive activation by fusion with *TPM3, TRK* can also be activated by fusion with *TPR, L7a* or *TFG* sequences on its 5' end. Two forms of the chimaeric TPR-TRK oncoprotein have been identified. In each, the amino-terminal sequences of TPR are fused to the TRK kinase domain. One is a protein of 55kDa consisting of 199 amino acids from TPR and 383 residues from TRK (Greco et al., 1992). The second form encodes a protein of 150kDa that contains additional TPR sequences fused to the same subset of TRK sequences present in TPM3-TRK (Barbacid, 1993). Since the genes for both proteins are located on chromosome 1, TPR-TRK is

generated by an intrachromosomal rearrangement. In the L7a-TRK fusion product, the amino-terminal sequence is contributed by the first 41 amino acids of the ribosomal large subunit protein L7a, while the carboxy-terminal sequence consists of the last 420 codons of TRK (Kozma et al., 1988; Ziemiecki et al., 1990). This 44kDa protein is localized to the cytoplasm and nucleoli (Ziemiecki et al., 1990). Finally, the *TRK* gene may fuse with an unknown 5' partner, termed TRK fused gene (*TFG*) owing to a t(1;3) chromosomal translocation. The product of this rearrangement, a 68kDa cytoplasmic protein, contains 199 amino acids from TFG and 471 residues from TRK (Greco et al., 1995).

1.2.3 RET fusion proteins

Most of the proto-oncogenes examined thus far are expressed during embryonic development, often in very defined spatial and temporal patterns that suggest functions in the growth and maturation of specific tissues (Dressler, 1994). Naturally occurring mutations in the receptor tyrosine kinase gene *RET*, are associated with the human diseases multiple endocrine neoplasia type 2A and familial medullary thyroid carcinoma (Mulligan et al., 1994; Mulligan et al., 1993; Takahashi and Cooper, 1987) In these patients the mutations occur in the extracellular domain of RET, where they alter

highly conserved cysteine residues. These mutations may result in the constitutive activation of RET by by-passing the need for ligand binding and/or facilitating dimerisation of unbound receptors (Dressler, 1994).

The *RET* gene is frequently activated in papillary thyroid carcinomas, either by an inversion, inv(10)(q11.2q21), that generates D10S170-RET, or by a chromosomal translocation, t(10;17)(q11.2;q23-24), that gives rise to RIα-RET, where the regulatory subunit of cyclic AMP-dependent protein kinase A (RIα) is fused to RET (Bongarzone et al., 1993; Ishizaka et al., 1989; Ishizaka et al., 1992; Ishizaka et al., 1990; Takahashi and Cooper, 1987). Both types of constitutively activated *RET* genes encode two isoforms. Under non-reducing conditions, the two RIα-RET isoforms occur as both homodimers and heterodimers (Bongarzone et al., 1993).

Mice deficient in RET completely lack neurons in the oesophagus, stomach and intestines, consistent with the observed expression of RET in the vagal neural crest cells that migrate from the hindbrain to populate the mesenteric plexus of the gut. The absence of gastrointestinal peristalsis in RET knockout mice is similar to the symptoms of the human congenital megacolon (Hirschsprung's disease), which has also been linked to the *RET*

locus on chromosome 10q11.2 (Angrist et al., 1993; Dressler, 1994; Lyonnet et al., 1993).

1.2.4 Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)

Nucleophosmin (NPM)

The human *NPM* (also known as B23, nutramin or NO38) cDNA encodes a nucleolar protein of 294 amino acids and has a reported molecular mass ranging from 32 to 38kDa. This ubiquitously expressed protein has a putative metal binding site, two acidic amino acid clusters, and two nuclear localisation signals (Chan et al., 1989) (figure 1.2). Under native conditions NPM is a hexamer of 220kDa consisting of 4α and 2β monomers organised in a head-to-tail fashion (Yung and Chan, 1987). Both the amino- and carboxy-terminal domains of NPM are essential for oligomerisation. Replacement of methionines at amino acid positions 5, 7, 9 with leucines or deletion of five amino acids at the carboxy terminus abolishes oligomerisation (Liu and Chan, 1991). On the other hand, Herrera and colleagues suggest that the carboxy terminus of NPM is not essential for oligomerisation (Herrera et al., 1996). NPM is expressed at higher levels in tumour cells than in normal cells, is highly phosphorylated during mitosis, and is a substrate for cdc2 (Chan et al., 1989; Feuerstein et al., 1990; Olson et al., 1974; Peter et al., 1990).

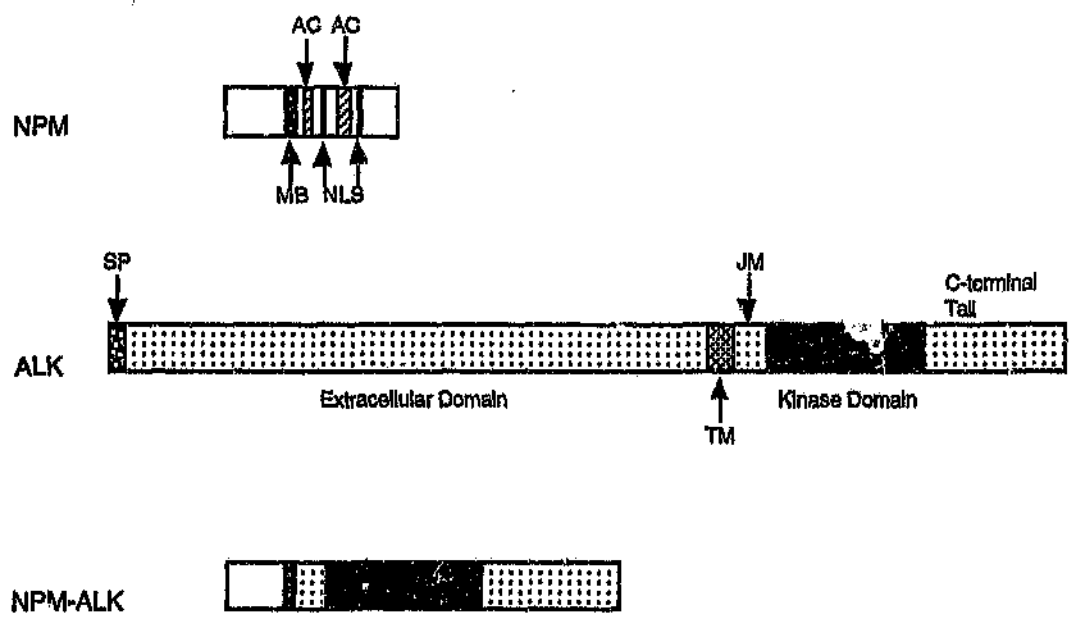


Figure 1.2. Schematic representation of the NPM, ALK and the chimaeric NPM-ALK oncoprotein. NPM which is composed of 294 amino acids, contains a putative metal binding domain (MB), two acidic regions (AC) and two nuclear localisation signals (NLS). ALK is a tyrosine kinase receptor consisting of 1620 amino acids with a signal peptide (SP), transmembrane domain (TM)and a juxtamembrane region (JM). The NPM-ALK chimaera is composed of the 118 amino-terminal amino acids of NPM fused to 562 residues from ALK.

Rat *NPM* exists as two alternatively spliced isoforms, these coding for peptides B23.1 (292 amino acids) and B23.2 (257 amino acids) (Chang and Olson, 1989). The two isoforms are expressed at different levels in tissues, B23.1 being more abundant. In addition, protein B23.1 is found predominantly in the nucleolus, while B23.2 appears to be present in the cytoplasm (Wang et al., 1993). Whereas B23.1 can bind RNA, single-stranded DNA and double-stranded DNA, B23.2 is unable to bind double-stranded DNA (Wang et al., 1994).

Although the function of NPM is not well defined, the protein is thought to be involved in ribosome assembly and transport (Chan and Chan, 1995). *In vitro* studies have demonstrated that NPM binds to RNA, DNA, the HIV REV protein, transcription factor YY1 and nucleolar protein p120 (Dumbar et al., 1989; Fankhauser et al., 1991; Feuerstein et al., 1990; Inouye and Seto, 1994; Valdez et al., 1994) This nucleolar phosphoprotein, which has a high affinity for peptides containing karyophilic sequences or nuclear localisation signals, was found to shuttle between the nucleolus and the cytoplasm (Borer at al., 1989; Szebeni et al., 1995). The above evidence suggests that NPM may have multiple functions.

Anaplastic lymphoma kinase (ALK)

The ALK tyrosine kinase receptor (1620 amino acids) has a 26 residue signal sequence, an extracellular domain of 1004 amino acids, a transmembrane region of 28 amino acids, a 64 residue juxtamembrane segment, a kinase domain of 254 amino acids, and an unusually long 244 residue carboxy-terminal tail (figure 1.2). After glycosylation, the ALK precursor (molecular mass of 177kDa) assumes its mature form as a 200kDa single-chain receptor (Morris et al., 1996).

ALK shows greatest amino acid similarity to insulin receptor subfamily members such as leukocyte tyrosine kinase (LTK; 64%), TRK (38%), ROS (37%), the β chains of the insulin-like growth factor receptor (37%) and insulin receptor (36%) (Morris et al., 1994). In addition, ALK contains paired autophosphorylation sites (Y^{1282} Y^{1283}) typical of members of the insulin receptor subfamily (Morris et al., 1996).

Four different *ALK* transcripts were found in tissues and cells from various sources. Messenger RNAs of 6.5 and 8kb were expressed to a high degree in rhabdomyosarcoma cell lines and in small intestine, and to a lesser degree in brain, colon and prostate. A 6kb transcript was identified in placenta and

foetal liver, while 4.4 and 6kb mRNAs were detected in testis. No *ALK* transcripts were apparent in haematopoietic cells (Morris et al., 1994). *In situ* hybridisation studies to localise the expression of mouse *ALK*, showed that *ALK* transcripts were limited to the central and peripheral nervous systems in this experimental model (Morris et al., 1996) (table 1.2).

NPM-ALK

Large-cell lymphomas constitute appr ..ately 25% of all paediatric and adolescent non-Hodgkin's lymphomas. About one-third of these tumours have a t(2;5)(p23;q35) chromosomal rearrangement that generates the NPM-ALK oncoprotein. This malignancy, which arises mainly from activated T-cells, invades and destroys normal lymph nodes, soft tissues, gastrointestinal tract, skin, lungs and bone marrow (Morris et al., 1994). By classical histochemical examination, the neoplastic cells have large irregular nuclei, prominent nucleoli and abundant cytoplasm. They express CD30/Ki-1, a cytokine receptor for a ligand related to tumour necrosis factor, and interleukin-2 (Downing and Morris, 1994; Morris et al., 1994). Cell lines with the t(2;5) express both the normal 1.6kb *NPM* transcript and a 2.4kb transcript, while those without the fusion gene express only the 1.6kb transcript. *NPM* presumably contributes an active promotor to drive

Age	ALK expression
Day-10 embryos	No transcripts
Day-11 embryos	Primitive spinal cord Cranial ganglia
Day-12 embryos	Spinal cord Cranial ganglia Periventricular zone around third ventricle
Day-14 embryos	Neuronal cells around third ventricle Olfactory nerves Ventral spinal cord
Day-15 (& older) embryos	Thalamic nuclei Cranial ganglia Sympathetic chain Ventral spinal cord
Newborn	Thalamic region
Adult	Thalamic region

Table 1.2. Tissue distribution of murine *ALK* transcripts within developing embryos. The pattern of *ALK* expression was determined by an *in situ* hybridisation technique (Morris et al., 1996).

expression of the *ALK* kinase domain, especially considering that ALK is not expressed in lymphoid cells (Morris et al., 1994).

Neither anaplastic morphology nor CD30 expression reliably identify NPM-ALK-positive lymphomas (Downing et al., 1995). In one study, cases without CD30 and/or NPM-ALK expression were classified as anaplastic large cell lymphomas (ALCLs); conversely cases without anaplastic morphology were found to express NPM-ALK (Downing and Morris, 1994). Waggot and co-workers found the t(2;5) in all Ki-1-positive cases of ALCLs, while Pulford and colleagues documented NPM-ALK expression in about half of their CD30-positive lymphoma cases (Pulford et al., 1996; Waggott et al., 1995). Herbst and associates identified NPM-ALK expressed predominantly in the ALCLs of young patients, with Elmberger and colleagues reporting that t(2;5) is unique to ALCLs (Elmberger et al., 1995; Herbst et al., 1995). NPM-ALK is also expressed in some patients with Hodgkin's lymphoma (Bullrich et al., 1994; Orscheschek et al., 1995). To resolve these conflicting observations, Sandlund and co-workers devised a model to explain the apparent overlap of ALCLs, Ki-1 positive large-cell non-Hodgkin's lymphomas and NPM-ALK containing lymphomas (Sandlund et al., 1994) (figure 1.3).

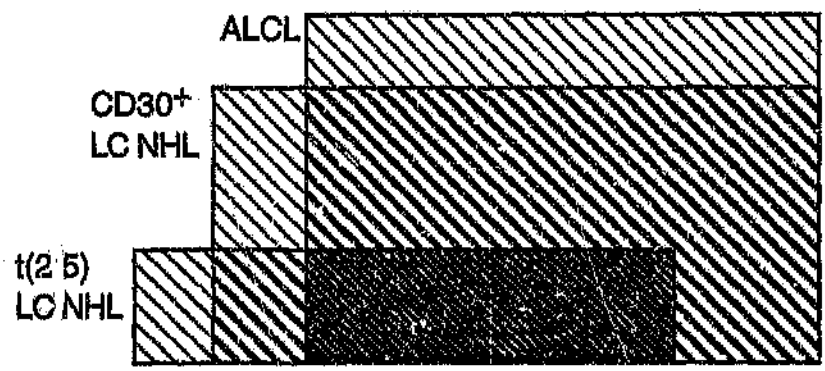


Figure 1.3. Schematic diagram illustrating the overlapping features of ALCLs, CD30+ large cell non-Hodgkin's lymphomas (LC NHLs) and NPM-ALK expressing LC NHLs (from Sandlund et al., 1994).

The NPM-ALK oncoprotein has 118 amino acids from NPM on its amino terminus and 562 residues from ALK on its carboxyl end (figure 1.2). Since the NPM amino terminus is retained in the chimaeric construct, and is necessary for NPM homo-oligomerisation, it is plausible to suggest that NPM-ALK may exist as either a homo-oligomer or in association with wild-type NPM (Liu and Chan, 1991). Indeed, homo-oligomerisation of NPM-ALK may constitutively activate the NPM-ALK kinase domain (figure 1.4). The two nuclear localisation signals found in NPM are deleted in the fusion protein, predicting a cytoplasmic localisation for the latter protein. Potential phosphorylation sites for protein kinase C and a putative metal-binding domain reside within the NPM portion of the chimaera, while possible binding sites for PI3'-kinase, IRS-1 and SHC are present in the ALK-derived sequences (Morris et al., 1994) (figure 1.5). The amino acid residues at positions 564 to 567 ($NPTY^{567}$) are suggested to be a SHC binding site, whereas the residues at amino acid positions 153 to 156 ($NPNY^{156}$) are predicted to be bind IRS-1 (Fujimoto et al., 1996).

NPM sequences have been found with fusion partners other than ALK, including the retinoic acid receptor α (*RARα*) in a patient with acute promyelocytic leukaemia and the t(5;17)(q32;q12) translocation. Two alternatively spliced forms of *NPM-RARα*, NPM_S-*RARα* and NPM_L-*RARα* were

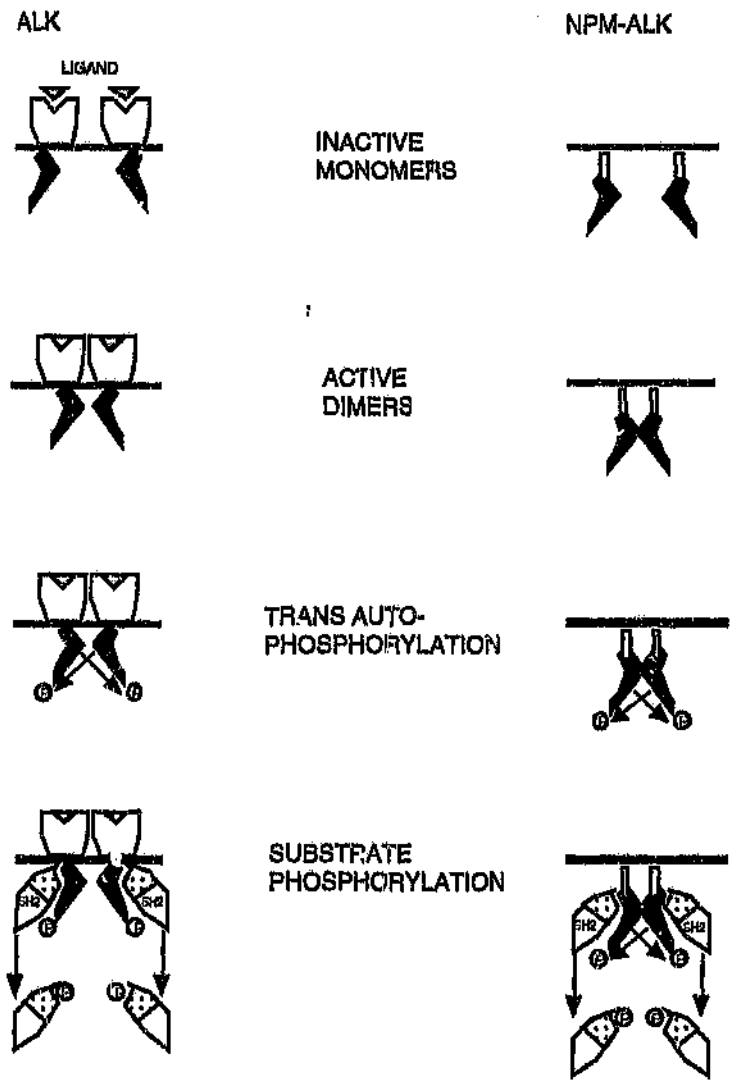


Figure 1.4. Model for the constitutive activation of NPM-ALK. Receptor tyrosine kinases are activated by ligand-mediated dimerisation which results in the trans-phosphorylation of receptor subunits, and the binding of the tyrosine phosphorylated receptor to intracellular substrates. NPM-ALK is thought to be a cytoplasmic protein that oligomerises through domains present in the amino terminus of NPM. This oligomerisation would be predicted to result in the constitutive activation of the of the ALK kinase domain and its interaction with and phosphorylation of downstream substrates (from Downing and Morris, 1994).

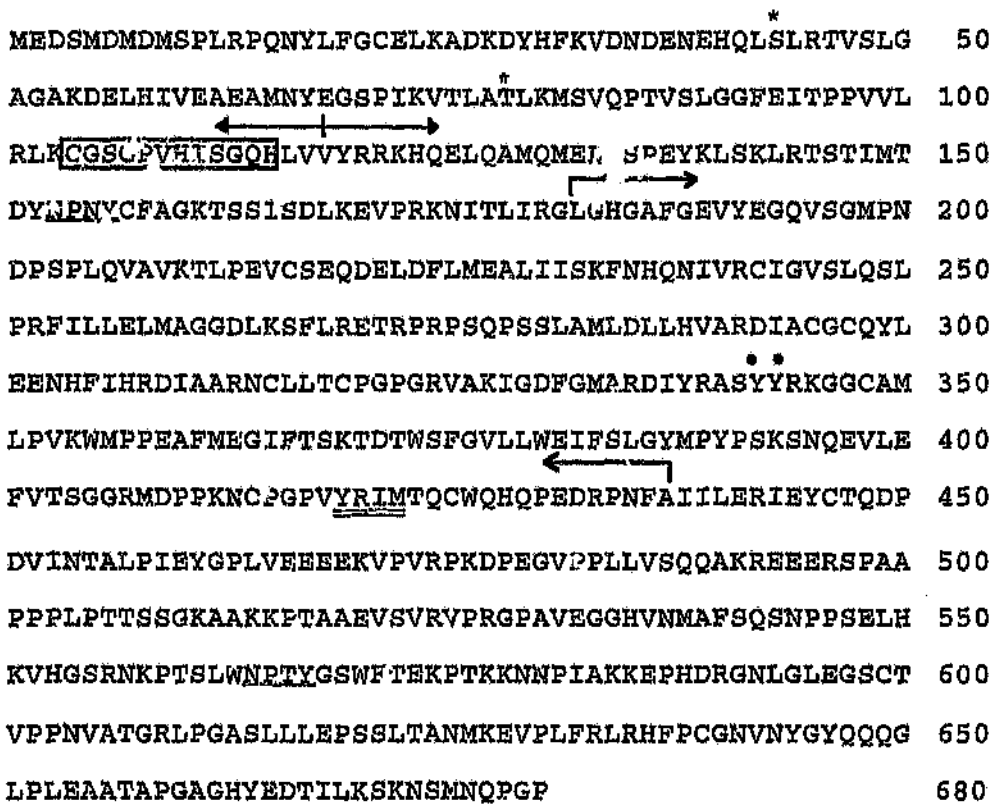


Figure 1.5. Deduced amino acid sequence of human NPM-ALK. Asterisks (*) depict putative protein kinase C phosphorylation sites, ↔ represents the NPM-ALK junction. ↱↰ indicates the boundaries of the kinase domain, • • shows the paired tyrosine autophosphorylation site typical of receptor tyrosine kinases in the insulin receptor subfamily. The boxed area is a putative metal binding domain. Underlined motifs represent possible IRS-1 and SHC binding sites, respectively. The double underlined amino acids indicate a putative PI3'-kinase binding region.

identified. Both proteins were expressed in the patient's leukaemic cells together with wild-type RARα (Redner et al., 1996). In cases of myelodysplastic syndrome and acute myeloid leukaemia with the t(3;5)(q25.1;q34) translocation, NPM is fused to myelodysplasia/myeloid leukaemia factor 1 (MLF1), a protein of unknown function. NPM-MLF1 is expressed in t(3;5)-positive leukaemia cells but wild-type MLF1 is not (Yoneda-Kato et al., 1996).

1.3 Objectives

Chromosomal rearrangements resulting in the constitutive activation of receptor tyrosine kinases have been shown to be oncogenic. A t(2;5) translocation found in about 10% of paediatric non-Hodgkin's lymphomas results in the expression of the nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) fusion protein. This chimaera consists of the amino terminal 40% of the nucleolar phosphoprotein, NPM, fused to the intracellular portion of ALK, a tyrosine kinase receptor. Despite some knowledge of structural features of NPM-ALK, very little is known about the manner in which this chimaeric protein transforms cells. Native NPM exists as an oligomer, so that oligomerisation of the NPM portion of the chimaera might be expected to activate ALK, thereby leading to malignant transformation. The work

outlined in this thesis is intended to test this hypothesis; deletion mutants of NPM-ALK were constructed and tested for oligomerisation, phosphorylation and cellular localisation in relation to transformation..

2. MATERIALS AND METHODS

2.1 Materials

Specific reagents used in this study were obtained from the sources indicated in the text. All other reagents were of analytical grade.

2.2 Cell culture

Six cell lines were used in this study: (1) Fisher rat 3T3 (Fr3T3), an immortalised but non-tumorigenic rat fibroblast line (provided by Morag Park, McGill University, Montreal, Canada), (2) NIH3T3, a mouse fibroblast line (provided by Martine Roussel, St. Jude Children's Research Hospital, Memphis, TN, USA), (3) 293T, an SV-40 transformed human kidney cell line (provided by Martine Roussel) (4) COS-7, an SV-40 transformed monkey kidney cell line (provided by David Shapiro, St. Jude Children's Research Hospital, Memphis, TN, USA), (5) SUP-M2, a human lymphoma cell line containing the t(2;5) (provided by Stephen Smith, University of Chicago Medical School, IL, USA), and (6) K562, a human leukaemia cell line (source unknown) (Lozzio and Lozzio, 1977; Morgan et al., 1989). Fr3T3, NIH3T3, 293T and COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (Mediatech, Herndon, VA, USA) supplemented with 10% foetal calf serum (FCS; BioWhittaker, Walkersville, MD, USA), whereas SUP-M2 and K562 cells

were grown in RPMI 1640 (BioWhittaker, Walkersville, MD, USA) supplemented with 20% FCS and 10% FCS, respectively. All cell lines were incubated at 37°C in a humidified incubator with 5% carbon dioxide. For experiments, adherent cells were trysinized with trypsin-ethylenediamine-tetraacetic acid (trypsin-EDTA; Life Technologies, Gaithersburg, MD, USA), quenched with complete medium and collected by centrifugation. Cells were then resuspended in fresh medium, counted on a haemocytometer using the trypan-blue exclusion test for viability, and plated onto tissue culture dishes as required (Patterson, 1979). Unless otherwise indicated, all experiments were carried out under the above-mentioned conditions.

2.3 DNA transfections

COS-7 cells were transiently transfected using the diethylaminoethyl-dextran (DEAE-dextran) method (Mammalian Transfection Kit; Stratagene, La Jolla, CA, USA). Briefly, $5X10^5$ cells were plated onto a 100mm culture dish one day prior to transfection. For transfection, 3µg of plasmid DNA was diluted with phosphate-buffered saline (PBS), and DEAE-dextran added to this. Cells were washed with PBS and the transfection mix added dropwise to the centre of the culture dish, which was gently swirled to distribute the solution evenly. Following a 15 minute incubation at room temperature, the solution

was removed, the cells washed with PBS, and fresh medium added. For experiments cells were harvested 72 hours post-transfection.

For immunofluorescence COS-7 cells were electroporated with the indicated DNA constructs. Approximately 10^7 cells were resuspended in 300µl of RPMI 1640 supplemented with 10% FCS and put into 0.4cm gap Gene Pulser cuvettes (Bio-Rad, Hercules, CA, USA). To this 5µg of DNA was added and allowed to rock at room temperature for 10 minutes. Cells were electroporated with a Bio-Rad Gene Pulser at 250V and 960µF. The cells were rocked for a further 10 minutes, after which they were plated onto coverslips in 35mm culture dishes at various dilutions, and incubated at 37°C for 24 to 36 hours.

2.4 Production of retroviral stocks

To optimise the efficiency of transformation, several batches of 2X BES-buffered saline (BBS) (50mM (N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES); Calbiochem, La Jolla, CA, USA), 280mM NaCl, 1.5mM Na_2HPO_4) ranging in pH from 6.92 to 7.00 were prepared and tested as previously described (Roussel et al., 1988). In brief, $3X10^5$ NIH3T3 cells in 35mm culture dishes were transfected using the calcium phosphate

technique, with 0.1µg of pSM feline sarcoma virus (FeSV) and 5µg of pBluescript carrier DNA. One day post-transfection each dish of cells was thinned out and maintained in Dulbecco's Modified Eagle's Medium supplemented with 5% FCS. The foci of transformed cells were scored 10 to 14 days after transfection. Only the buffer yielding the highest transformation efficiency, that is the most foci, was used in subsequent experiments.

Helper-free retrovirus was generated by transfecting 293T cells with both Ψ2 packaging DNA (provided by Owen Witte, HHMI, University of California, Los Angeles, CA, USA) and construct DNA in retroviral vector, using the calcium phosphate precipitation method (Graham and Van der Eb, 1973; Pear et al., 1993) (pers. commun. Martine Roussel, St. Jude Children's Research Hospital, Memphis, TN, USA). A day prior to transfection, 293T cells were seeded out at $2x10^6$ cells per 100mm plate in 10ml of medium. For transfection, 20µg of each of Ψ2 and construct DNA were made up to a final volume of 450µl with deionised water. To this 50µl of 2.5M calcium chloride was added, followed by the addition of 500µl of 2X BBS. This solution was vortexed and incubated at room temperature for 20 to 30 minutes, after which the calcium phosphate-DNA mix was added dropwise to the medium in the plates containing the 293T cells. The transfected cells

were incubated for approximately 18 hours in the presence of 8% carbon dioxide, after which they were rinsed with PBS and fresh medium was added. After a further 6 hours incubation, a minimal amount of fresh medium was added (usually 3 to 4ml). Collection of retroviral stocks was commenced 30 hours post-transfection and continued every 6-12 hours for a total of 36 hours. Viral supernatants which were kept on ice at all times, were pooled, filtered through 0.45µm pore size filters and used to infect Fr3T3 cells. All solutions used for transfections, excluding the DNA, were filter sterilised through 0.2µm pore size filters.

2.5 Retroviral Infections

Fr3T3 cells were seeded at a density of $2X10^5$ cells per 100mm plate 24 hours prior to infection. For infection the medium was removed from cells, and 2ml of virus supernatant containing 8µg/ml polybrene (Sigma, St. Louis, MO, USA) was added and allowed to incubate in the presence of 8% carbon dioxide for 3 to 4 hours. Growth medium was then added and cells incubated for a further 72 hours. At this time Fr3T3 cells were used for transformation assays, focus formation, G418 selection and for the derivation of clonal cell lines (see sections 2.6 and 2.7).

2.6 Transformation assays

One day prior to soft agar cloning, 2ml of bottom agar was poured into 35mm culture dishes. Noble agar (Difco Laboratories, Detroit, MI, USA) was made to 1.2% in deionised water, autoclaved and allowed to cool to 45°C. This was diluted 1:1 with prewarmed 2X complete Iscove's Modified Dulbecco's Medium (BioWhittaker, Walkersville, MD, USA) yielding a 0.6% bottom agar in 1X Iscove's Modified Dulbecco's Medium supplemented with 15% FCS. Fr3T3 colony formation in soft agar was measured by plating $5X10^5$ or $2X10^4$ cells in 0.3% noble agar in Iscove's Modified Dulbecco's Medium containing 15% FCS, onto the 0.6% bottom agar. Colony formation was assessed 10 to 14 days after plating.

2.7 Focus formation, G418 selection and derivation of clonal cell lines

Infected Fr3T3 cells were plated out in duplicate at $2X10^6$ cells per 100mm culture dish. On the following day complete medium supplemented with 800µg/ml of G418 (Geneticin; Life Technologies, Gaithersburg, MD, USA) was added to one of the dishes containing cells. Both sets of cells were maintained in culture for 10 to 14 days. At this stage the unselected plates were stained with Giemsa (see section 2.8), while the G418 selected plates were used for the derivation of single-cell clones. Briefly, sterile glass cloning

rings were placed onto plaques of cells originating from a single cell, with sterile petroleum jelly. The cells within the cylinder were trypsinised and further propagated. Alternatively, where clones had formed in soft agar, these were plucked with a pipette, trypsinised and also allowed to multiply further.

2.8 Giemsa staining

Cells were rinsed with PBS and stained with Giemsa (CMS Stain Pack; Biochemical Sciences, Swedesboro, NJ, USA). The stain was left on the cells for 1.5 minutes, after which an equal volume of buffer 2 was added and left for an additional 1.5 minutes. Cells were rinsed with PBS and allowed to dry in an inverted position.

2.9 Polymerase chain reaction (PCR)

TPR-ALK and *NPM-MET* were generated using a three-oligonucleotide PCR method (Davis et al., 1994). Δ^{MB}*NPM-ALK* and $\Delta^{65\text{-}103}$*NPM-ALK* were constructed using the thermal cycled fusion method (Kahn et al., 1990). $^{M57/9L}$*NPM-ALK* and ^{K210R}*NPM-ALK* were generated by PCR using oligonucleotide primers containing engineered nucleotide substitutions designed to alter selected codons. Other constructs were generated by

PCR-based mutagenesis. Reaction mixtures for three-oligonucleotide PCRs contained 0.5ng of each of the two templates, 100ng of both the sense and antisense primers, 20 to 300ng of the hybrid primer, 400µM of each of the four deoxynucleotide triphosphates (Pharmacia, Piscataway, NJ, USA), 1X *Pfu* polymerase buffer and 2.5U of recombinant *Pfu* polymerase (Stratagene, La Jolla, CA, USA) in 50µl reactions. The reaction mixtures for all other PCRs contained 200ng to 1µg of template, 0.5µM of each primer, 200µM of each of the four deoxynucleotide triphosphates, 1X *Pfu* polymerase buffer and 2.5U recombinant *Pfu* polymerase in 50µl reactions. Templates were denatured at 95°C for 5 minutes, and thirty cycles, each consisting of 1 minute denaturation at 95°C, 1 minute of annealing at 50°C and 1.5 minutes extension at 72°C were conducted.

2.10 Ligations and transformations

Ligations were performed using a DNA ligation kit (Stratagene, La Jolla, CA, USA). Reactions were set up containing an equimolar ratio of vector to insert, 1mM rATP, 1X ligase buffer and 4U of T4 DNA ligase in a total volume of 10µl. Ligation was allowed to proceed overnight at 12°C. Subsequently, the ligation mix was made up to 25µl with water and the DNA precipitated with 10 volumes of n-butanol. The speedvac dried pellet was

redissolved in 10µl of water. ElectroMAX DH10B bacteria (20µl) (Life Technologies, Gaithersburg, MD, USA) were pipetted into a chilled 0.2cm gap Gene Pulser/E. coli pulser cuvette (Bio-Rad, Hercules, CA, USA) and half of the ligation mix added. Electroporations were performed at 2500V, 200Ω and 25µF. Immediately after electroporation 0.5ml of SOC (20g/l tryptone, 5g/l yeast extract, 0.5g/l sodium chloride, 2.5mM potassium chloride, 20mM magnesium chloride and 20mM glucose) was added, and the bacteria allowed to recover at 37°C for one hour. Since all the vectors used in this study contain the ampicillin resistance gene the DH10B cells were plated out on LB agar plates (10g/l tryptone, 5g/l yeast extract, 10g/l sodium chloride and 15g/l agar) containing 50µg/ml of ampicillin.

To facilitate cloning into the *Eco*RI or *Hind*III site of the retroviral vector pSRαMSVtkneo (section 2.14), linkers were ligated to blunted cDNAs. All cDNAs with 5' overhangs were filled in using Klenow enzyme (Random Primed DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN, USA). Subsequently *Eco*RI or *Hind*III linkers were added by incubating 500ng of DNA insert, 2µg of phosphorylated linkers, 1mM rATP, 1X ligase buffer and 12U of T4 DNA ligase in a total volume of 30µl at 12°C overnight. The DNA in the linker mix was precipitated by the addition of 300mM sodium acetate, pH 5.2, and two volumes of ethanol. This was centrifuged and the pellet

redissolved in 1X TE (10mM Tris-HCl, pH 8 and 1mM EDTA). The cDNA insert with added linkers was digested with the appropriate restriction enzyme and purified using the Geneclean II Kit (Bio 101, Vista, CA, USA), and utilised for subsequent ligations.

2.11 Plasmid preparations

Bacteria expressing construct DNAs were grown in LB (10g/l tryptone, 5g/l yeast extract and 10g/l sodium chloride) containing 50µg/ml of ampicillin. All plasmids were isolated from bacteria using Qiagen Plasmid Kits (Qiagen, Chatsworth, CA, USA).

2.12 DNA sequencing

Reading frames of the cDNA constructs were confirmed by *in vitro* transcription and translation (see section 2.17) followed by SDS-PAGE to demonstrate a peptide of predicted size. The possibility of errors introduced by PCR was excluded by sequencing. The dideoxy chain termination method (Sanger et al., 1977) using the Sequenase Version 2.0 kit (USB, Cleveland, OH, USA) was employed. Five µg of plasmid DNA in a volume of 8µl was denatured by the addition of 2µl of 2M NaOH and heating to 85°C for 5 minutes. The DNA was put on ice and neutralised by adding

1.4µl of 3M sodium acetate, pH 5.2. To this 0.5µl of single-stranded DNA binding protein (Stratagene, La Jolla, CA, USA), 50ng primer and 40µl of ethanol were added. Following the placement at -20°C for at least 15 minutes, the denatured DNA-primer mix was centrifuged, and the pellet resuspended in 7µl of water. Annealing of primer to DNA was performed at 37°C for 20 to 30 minutes. The labelling reaction was commenced by the addition of 2µl of 5X Sequenase buffer, 1µl of 0.1M dithiothreitol, 2µl of 1X dGTP labelling mix, 1µl of [α-^{35}S]dATP (1000Ci/mmol; Amersham, Arlington Heights, IL, USA) and 2µl of Sequenase diluted 1:8 in enzyme dilution buffer, and incubated at room temperature for 5 minutes. Termination reactions were initiated when 3.5µl of the labelling reaction was added to each of four tubes containing 2.5µl of one of the four dideoxynucleotide triphophates (ddATP, ddCTP, ddGTP or ddTTP), and incubated at 37°C for 3 minutes. Four µl of stop solution was added to each reaction. Prior to loading, samples were heated to 100°C and placed on ice.

Gel-Mix 8 (Life Technologies, Gaithersburg, MD, USA) was used to prepare sequencing gels. Gels were run at 80W in 0.5X TBE (45mM Tris-borate and 1mM EDTA, pH8).

2.13 Primers

All primers were synthesised by the Centre of BioTechnology at St. Jude Children's Research Hospital, Memphis, TN, USA. Nucleotides that were altered from the original sequence, so as to introduce a restriction site, mutation or premature stop codon, are indicated in lower case letters. A short explanation of each primer is included to make its origin clear. The sequences of clones 7-8, 20λ-1, 5'-250λ-1 and *TPR-MET* in pGEM-9Zf(-) are illustrated in figures 2.1, 2.2, 2.3 and 2.4, respectively, and their derivation is explained under plasmid construction (see section 2.14).

2.13.1 PCR primers

3'-Δ38 The reverse complement of the sequence found at positions 645-669 in clone 7-8 (5' GTTGTAGTCGGTCATGATGGTCGAGGT 3').

3'-ΔC154 The reverse complement of sequences at positions 1777-1818 in clone 7-8 (5' CATATTCACGTGTCaagCTTCCACGGCCGGCCCTCaAGGGAC 3'). A *Hind*III site and a stop codon were engineered.

3'-ΔC42MOD Reverse complement of nucleotides at positions 2113-2157 in 7-8 in pBS (5' CAAGCCCTGTTGCTGGTAGCCaagcTTGACATTCCCtCAAGGGAA 3'). A *Hind*III site and a stop codon were engineered.

3'END RV-1 Corresponds to nucleotides at positions 1943-1969 in clone 7-8 (5' ATCCTATAGCAAAGAAGGAGCCACACG 3').

3'END RV-2 Reverse complement of sequences at positions 2227-2261 in clone 7-8 (5' GTGTGCGACCGAtaTCAGGGCCCAGGCTGGTTCAT 3'). An *Eco*RV site was engineered.

3'LYS-24 Reverse complement of nucleotides at positions 1267-1290 in clone 7-8 (5' CATGAAGGCCTCTGGGGGCATCCA 3').

3'METH-33 Reverse complement of sequences at positions 487-519 in clone 7-8 (5' ACCACACTTCAACCTTAAGACCACTGGTGGTGT 3').

5'-Δ38 Polylinker of pBluescript and 5' untranslated sequences at positions 99-124 in clone 5'-250λ-1 (5' TAGTGGATCCCCCG[illegible]CGCCTTCTCT 3').

5'-ΔC42 Nucleotides at positions 1885-1920 in clone 7-8 (5' AGCTTGTGG AACCCAACGTACGGCTCCTGGTTTACA 3').

5'-ΔC154(+) Sequences at positions 1438-1460 in clone 7-8 (5' AAGAACTGCCCTGGGCCTGTATA 3').

5'END RV-1 Nucleotides at positions 159-194 in clone 7-8 (5' TATCCcCGggCGCCTTCTCTCCTACCTAAGTGCGTG 3'). A *Sma*I site was engineered.

5'END RV-2 Reverse complement of sequences at positions 531-554 in clone 7-8 (5' ACTAAGTGCTGTCCACTAATATGC 3').

5'LYS-60 Nucleotides at positions 784-843 in clone 7-8 (5' CAGGTGTCCGGAATGCCCAACGACCCAAGCCCCCTGCAAGTGGCTGTGAgGACGCTG CCT 3'). The lysine at amino acid position 210 was mutated to an arginine.

5'METH-60 Nucleotides at positions 181-240 in clone 7-8 (5' TACCgAAtTcCGTGCCCCCACCCGATGGAAGATTCGcTGGACcTGGACcTGAGCCCCC TG 3'). In addition to engineering an *Eco*RI site, the methionines at amino acid positions 5, 7 and 9 were mutated to leucines.

AS-3'ALK Reverse complement of sequences at positions 763-789 in clone 7-8 (5' CACCTGGCCTTCATACACCTCCCCAAA 3').

AS-3'MET-APAI Reverse complement of nucleotides at positions 925-956 in *TPR-MET* in pGEM-9Zf(-) (5' CCTCTTCCTATGACTTCATTGAAATGCACAAT 3').

AS-3'UT-MET-H3-SALI Reverse complement of untranslated sequences at positions 1863-1905 in *TPR-MET* in pGEM-9Zf(-) (5' CAAAGTGTCGACTGAA GCTTTGACATAGTACTAGCACTATGAT 3'). Both a *Sal*I and a *Hind*III site were introduced into the primer.

AS-BKPT-NPM-MET The reverse complement of nucleotides from the portion of *NPM* immediately 5' of the breakpoint, and the region of *MET* immediately 3' of the fusion junction. The italisised nucleotides are derived from *NPM* at positions 536-556 (clone 7-8) while the other nucleotides are from *MET* at positions 725-745 (*TPR-MET* in pGEM-9Zf(-)) (5' GAGATGAATTAGCAAACTGAT *CTACTAAGTGCTGTCCACTAA* 3').

Δ38-AS The reverse complement of Δ38-S (5' ATGCACTGGCCCTGAACC ACATGCCTCTGCTTCAACAATGTG 3').

Δ38-S The nucleotides at positions 376-396 and 514-534 in clone 7-8 (5'CACATTGTTGAAGCAGAGGCATGTGGTTCAGGGCCAGTGCAT 3').

ΔMB-B The reverse complement of ΔMB-C (5' CTTCCGGCGGTACACTACT AACTTCAACCTTAAGACCACTGG 3')

ΔMB-C Sequences at positions 493-513 and 550-570 in clone 7-8 (5' CCAGTGGTCTTAAGGTTGAAGTTAGTAGTGTACCGCCGGAAG 3').

S-3'MET-RV Nucleotides at positions 1688-1717 in *TPR-MET* in pGEM-9Zf(-) (5' CTGAACTGGTGTCCCGGATATCAGCGATCT 3').

S-5'UT-NPM-H3 Untranslated sequences at positions 163-189 in clone 7-8 (5' TCCGTCaagCTTCTCTCCTACCTAAGT 3'). A *Hind*III site was engineered.

S-5'UT-TPR-SMAI Untranslated sequences at positions 254-285 in *TPR-MET* in pGEM-9Zf(-) (5' CTACCCCGggGCCCGCTGCCACTGGGTCCCT 3'). A *Sma*I site was engineered.

S-BKPT-TPR-ALK Nucleotides from the portion of *TPR* immediately 5' of the breakpoint, and the region of *ALK* immediately 3' of the fusion junction. The italisised sequences are derived from *TPR* at positions 704-724 (*TPR-MET* in pGEM-9Zf(-)), whereas the other nucleotides are from *ALK* at positions 557-577 (clone 7-8) (5' *AAGAACTTGAATACTTAACAG* TGTACCGCCGCAAGCACCAGG 3').

S-D64-NPM Sequences from positions 175-204 in clone 7-8 (5' CTCTCCTAC CTAcccGgGTGCCCCCACCCGATGAATTACGAAGGCAGTCCAATTAAAGTA 3'). A *Sma*I site was engineered.

2.13.2 Sequencing primers

AS-ALK-24 Reverse complement of sequences at positions 855-878 in clone 7-8 (5' AGGAAATCCAGTTCGTCCTGTTCA 3').

AS-KR-21 Reverse complement of nucleotides at positions 995-1015 in clone 7-8 (5' TCTCTCGGAGGAAGGACTTGA 3').

AS-MET-901-922 Reverse complement of sequences at positions 986-1007 in *TPR-MET* in pGEM-9Zf(-) (5' TTCTTGCCATCATTGTCCAACA 3').

AS-ML-23 Reverse complement of sequences at positions 607-629 in clone 7-8 (5' TTGCTCAGCTTGTACTCAGGGCT 3').

pBS-TPR Reverse complement of nucleotides at positions 232-257 in *TPR-MET* in pGEM-9Zf(-) (5' GTAGAAGCGGAGAAGAAAGGCGAAGA 3').

pGEX-NPM Reverse complement of sequences at positions 376-396 in clone 7-8 (5' TGCCTCTGCTTCAACAATGTG 3').

pQE-NPM-3' Sequences at positions 405-432 in clone 7-8 (5' CGAAGGCAG TCCAATTAAAGTAACACTG 3').

pUC M13 REVERSE In the polylinker of pBluescript at positions 3-21 in clone 7-8 (5' GGAAACAGCTATGACCATG 3').

S-3'MET Nucleotides at positions 1586-1616 in *TPR-MET* in pGEM-9Zf(-) (5' TGCAAGGGAGAAGACTCCTACAACCCGAATA 3').

S-ΔC-24 Nucleotides at position 1378-1401 in clone 7-8 (5' AGCAAAAGC AACCAGGAAGTTCTG 3')

S-KR-22 Sequence at positions 668-689 in clone 7-8 (5' ACTACTGCTTTGCT GGCAAGAC 3').

S-MB-23 Sequence at positions 392-414 in clone 7-8 (5' AGGCAATGAATT ACGAAGGCAGT 3').

SP6 Sequence at positions 23-41 in *TPR-MET* in pGEM-9Zf(-) (5' GATTTAGG TGACACTATAG 3').

T7 The reverse complement of nucleotides at positions 2096-2115 in *TPR-MET* in pGEM-9Zf(-) and positions 2638-2657 in clone 7-8 in (5' TAATACGACTCACTATAGGG 3').

TPR-3' Nucleotides at positions 619-641 in *TPR-MET* in pGEM-9Zf(-) (5' GCCATTCAGAGCCAATTTACAAG 3').

2.14 Plasmid construction

NPM-ALK: Clone 7-8 is the *NPM-ALK* cDNA with its 5' and 3' untranslated (UT) ends cloned into the *Eco*RI site of pBluescript SK(+) (Stratagene, La Jolla, CA, USA) (figure 2.1). Since one of the *Eco*RI sites was destroyed during cloning, the 2.4kb *NPM-ALK* cDNA was excised from clone 7-8 using *Sma*I/*Eco*RV to yield template for PCRs. The 5'UT and 3'UT ends of clone 7-8 were trimmed prior to cloning into the retroviral vector pSRαMSVtkneo (provided by Owen Witte, HHMI, University of California, Los Angeles, CA, USA) (Muller et al., 1991). All of the 3'UT end was trimmed, by engineering an *Eco*RV site, using PCR primers 3'END RV-1 and 3'END RV-2, and the 2.4kb *NPM-ALK* cDNA as template. The resulting 319bp PCR product which corresponds to the nucleotides encoding amino acids 580-680 of *NPM-ALK* and 16bp of 3'UT sequence, was digested with *Bst*EII/*Eco*RV to yield a 273bp fragment. Similarly, clone 7-8 was digested with *Bst*EII/*Eco*RV to release a 610bp insert, which was replaced with the 273bp fragment. The resulting construct was designated 20λ-1 (figure 2.2). The 5'UT end was pruned by engineering a *Sma*I site immediately 5' of the Kozak sequence using PCR primers 5'END RV-1 and 5'END RV-2, and the 2.4kb *NPM-ALK* cDNA as template. The 396bp PCR fragment which corresponds to the 36bp immediately upstream of the start codon and the nucleotides

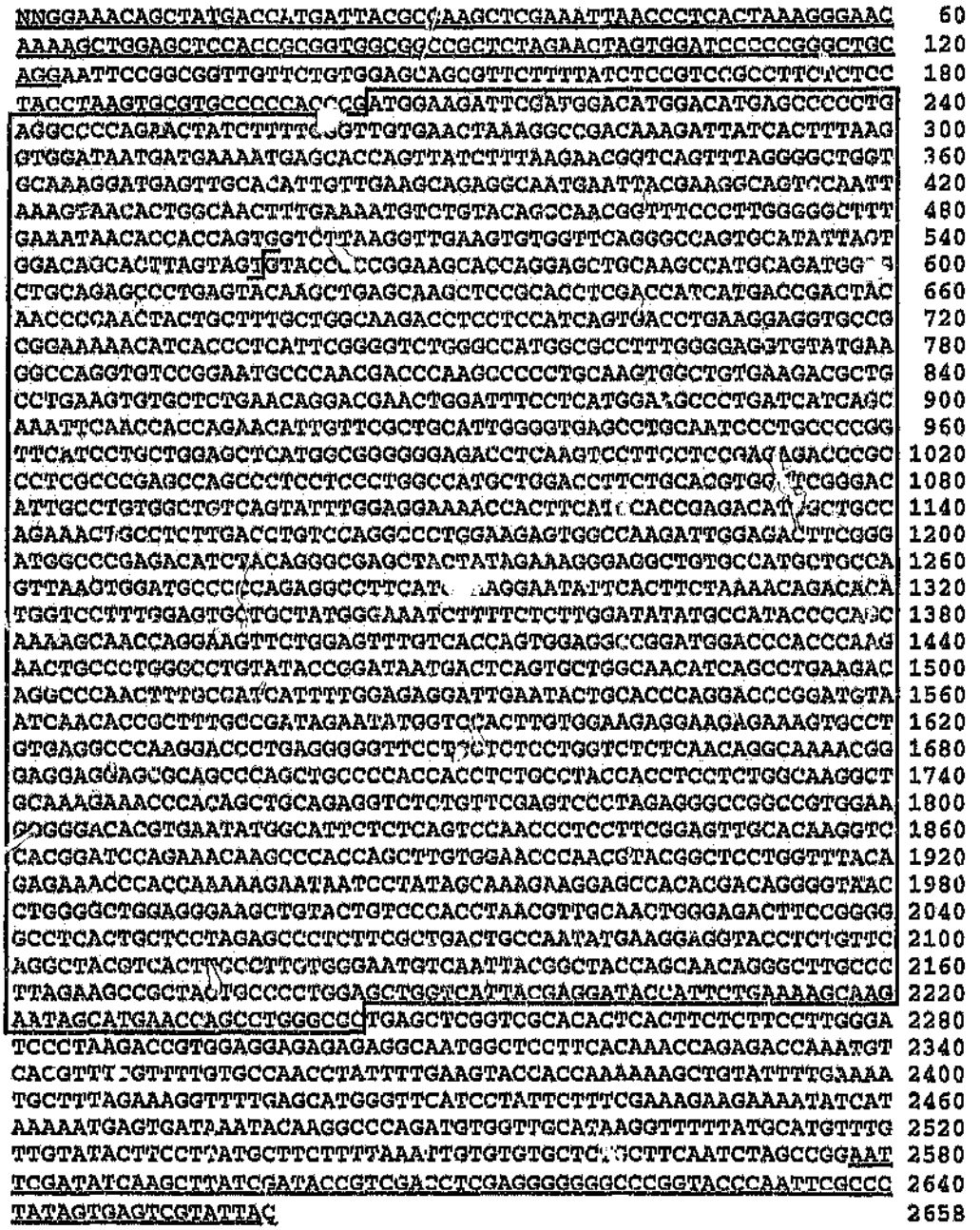


Figure 2.1. Clone 7-8. This clone is the *NPM-ALK* cDNA with its 5' and 3' untranslated ends cloned into the *Eco*RI site of pBluescript SK(+). The shaded area represents the coding sequence; underlined sequences correspond with pBluescript polylinker; and remaining sequences are the 5' and 3' untranslated ends. (Genbank accession number HSU04946).

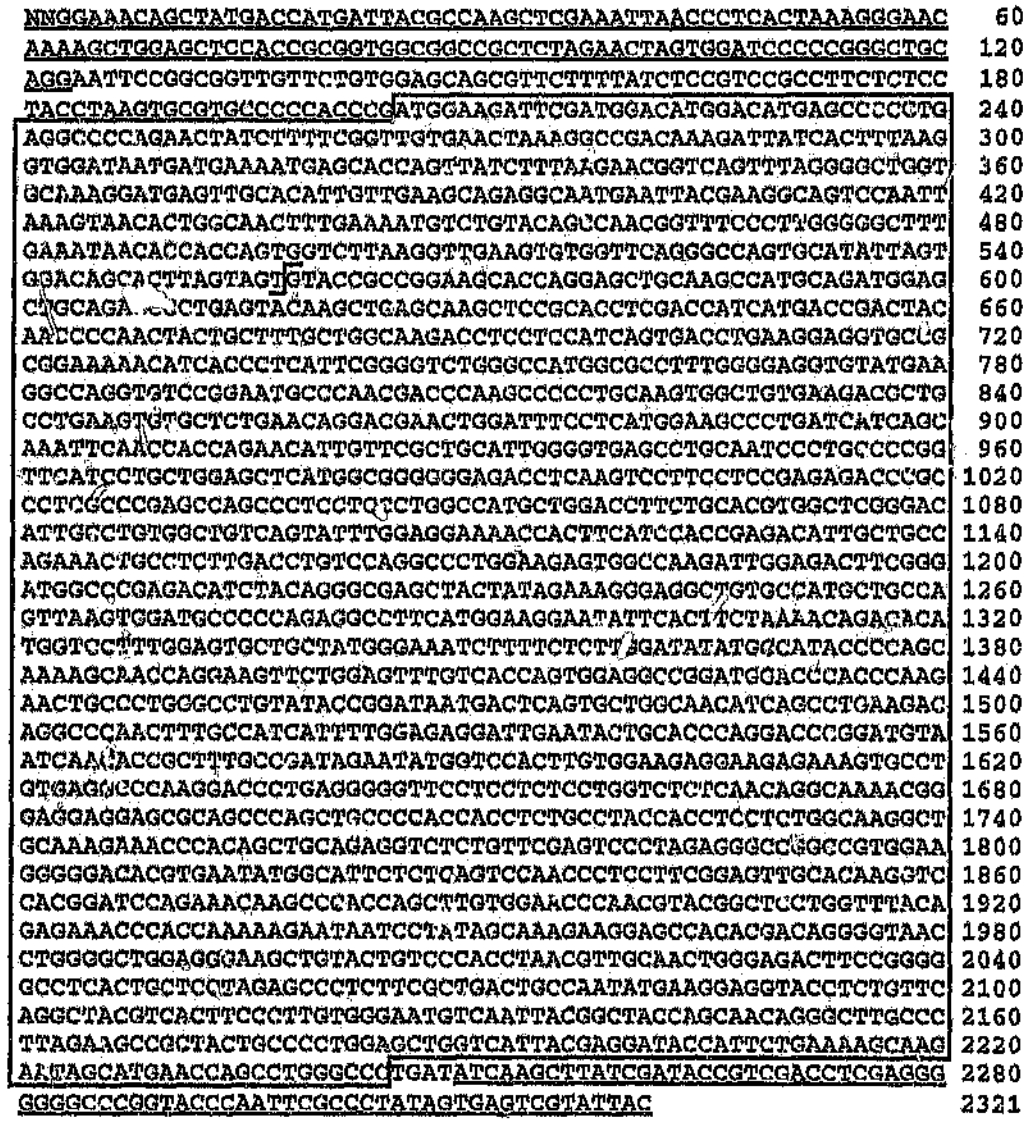


Figure 2.2. Clone 20λ-1. The 3' untranslated sequence from clone 7-8 was trimmed. The shaded area represents the coding sequence; underlined sequences correspond with pBluescript polylinker; and remaining sequences are the 5' and 3' untranslated ends.

encoding the first 117 amino acids of *NPM-ALK*, was digested with *SmaI/AflII* to produce a 334bp fragment. Clone 20λ-1 was also digested with *SmaI/AflII* to liberate a 388bp insert, which was substituted with the 334bp fragment. This construct which was termed 5'-250λ-1 (figure 2.3), was digested with *SmaI/Eco*RV, and *Hind*III linkers (New England BioLabs, Beverly, MA, USA) were added. This 2083bp insert was subsequently cloned into the *Hind*III site of pSRαMSVtkneo (prepared by Mark Kirstein in the laboratory of S.W. Morris).

TPR-MET: A 1.99kb *Eco*RI *TPR-MET* insert with its 5'UT and 3'UT ends (provided by Morag Park, McGill University, Montreal, Canada) was cloned into the *Eco*RI site of pSRαMSVtkneo. The 1.99kb insert was used as template for PCRs.

TPR-ALK: This chimaera was generated using both the 2.4kb *NPM-ALK* and 1.99kb *TPR-MET* cDNAs as templates, and PCR primers S-5'UT-TPR-SMAI, S-BKPT-TPR-ALK and AS-3'-ALK. The 703bp PCR product which encompasses 44bp of 5'UT sequence and the nucleotides encoding the entire coding region of the *TPR* portion in *TPR-MET* and the nucleotides encoding amino acids 118-195 of *NPM-ALK*, was digested with *SmaI/NarI* to yield a 665bp fragment. The *SmaI/NarI* digestion of clone 20λ-1 released a 645bp insert which was replaced with the 665bp fragment. This construct was sequenced using primers pUC M13 REVERSE, pBS-TPR, TPR-3' and

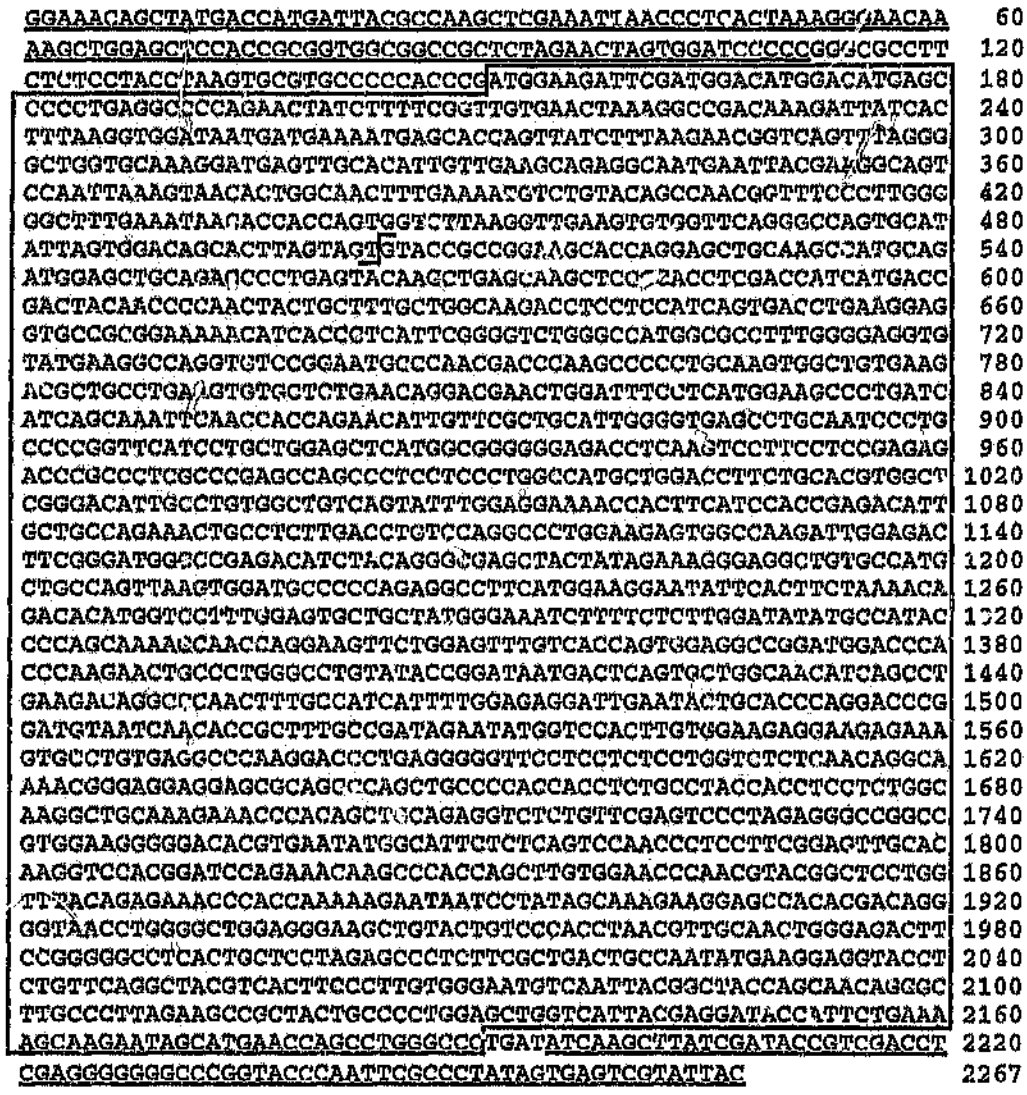


Figure 2.3. Clone 5' 250λ-1. Most of the 5' untranslated end was removed from clone 20λ-1. The shaded area represents the coding sequence; underlined sequences correspond with pBluescript polylinker; and remaining sequences are the 5' and 3' untranslated ends.

AS-ALK-24. The 2155bp *TPR-ALK* was excised with *Sma*I/*Eco*RV, *Eco*RI linkers (New England BioLabs, Beverly, MA, USA) were added, and the chimaeric construct ligated into the *Eco*RI site of pSRαMSVtkneo.

NPM-MET: The 1.99kb *Eco*RI *TPR-MET* cDNA insert was cloned into the *Eco*RI site of plasmid vector pGEM-9Zf(-) (Promega, Madison, WI, USA) (figure 2.4). To generate an *NPM-MET* chimaera with the 5'UT end trimmed, the same DNA templates were utilised as for the construction of *TPR-ALK*, together with PCR primers S-5'UT-NPM-H3, AS-BKPT-NPM-MET and AS-3'-MET-APAI. The 626bp PCR fragment which corresponds to 42bp of 5'UT sequence and the nucleotides encoding the entire coding region of the *NPM* portion in *NPM-ALK* and the nucleotides encoding amino acids 143-220 of *TPR-MET*, was cut with *Hind*III/*Apa*I to yield a 557bp fragment. *TPR-MET* in pGEM-9Zf(-) was digested with *Hind*III/*Apa*I to liberate an 956bp insert which was replaced with the 557bp fragment (designated clone 626). Clone 626 was sequenced using primers SP6, pGEX-NPM, pQE-NPM-3' and AS-MET-901-922. To trim the 3'UT end, the 1.99kb *TPR-MET* cDNA template and PCR primers S-3'MET-RV and AS-3'UT-MET-H3-SALI were used. The resulting 218bp PCR product which encompasses the nucleotides encoding amino acids 464-523 of *TPR-MET* and 39 nucleotides of 3'UT sequence, was digested with *Eco*RV/*Sal*I to yield a 188bp fragment. Clone 626 was digested with *Eco*RV/*Sal*I to release 379bp, which was substituted with the

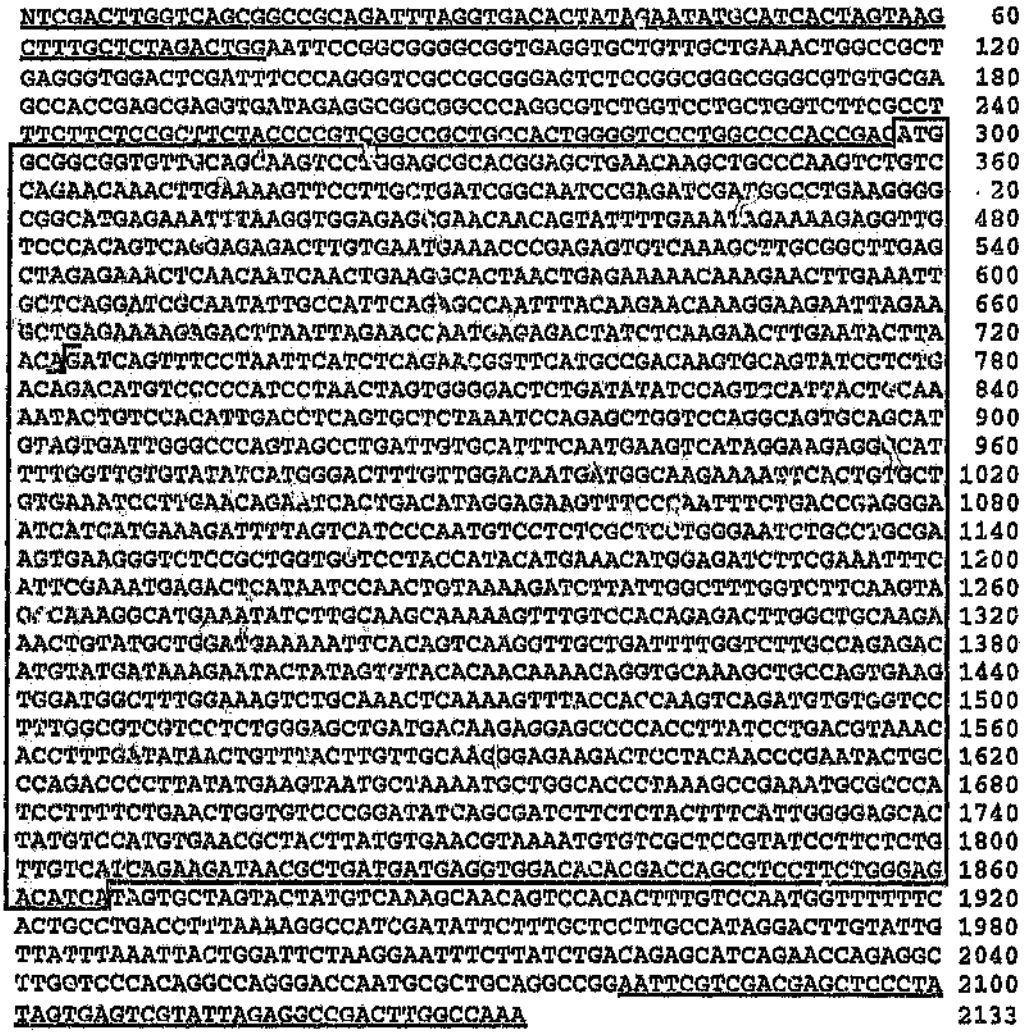


Figure 2.4. *TPR-MET* in pGEM-9Zf(-). The *TPR-MET* cDNA with its 5' and 3' untranslated ends intact was cloned into the *Eco*RI site of pGEM-9Zf(-).The shaded area represents the coding sequence; underlined sequences correspond with pBluescript polylinker; and remaining sequences are the 5' and 3' untranslated ends. (Genbank accession number HSU19343).

188bp fragment. This construct was sequenced utilising primers S-3'MET and T7. This 1549bp chimaeric construct was digested with *Hind*III and subcloned into pSRαMSVtkneo.

$^{M6/7/9}$*NPM-ALK*: Methionines at positions 5, 7 and 9 were mutated to leucines. The *NPM-ALK* cDNA was excised from pSRαMSVtkneo/*NPM-ALK* with *Hind*III and cloned into pBluescript SK(+) (clone pBS/*NPM-ALK*). PCR was performed using the 2.4kb *NPM-ALK* cDNA as template with primer pair 5'-METH-60 and 3'-METH-33. The 339bp PCR fragment which corresponds to 24bp of 5'UT sequence and the nucleotides encoding the first 105 amino acids of *NPM-ALK*, was digested with *Eco*RI/*Afl*II to produce a 316bp fragment. Similarly, clone pBS/*NPM-ALK* was digested with *Eco*RI/*Afl*II to liberate a 356bp insert which was replaced with the 316bp fragment. The construct was sequenced using the pUC M13 REVERSE and AS-ML-23 primers. This 2057bp mutant construct was cloned into the *Eco*RI/*Hind*III sites of pSRαMSVtkneo.

^{K210R}*NPM-ALK*: The lysine residue at position 210 was mutated to an arginine. PCR primers 5'-LYS-60 and 3'-LYS-24, and the 2.4kb *NPM-ALK* cDNA template were used to generate a 507bp product. This PCR product which corresponds to the nucleotides encoding amino acids 194-362 of *NPM-ALK*, was digested with *Bsp*EI/*Stu*I to yield a 492bp fragment. At the same time clone 5'-250λ-1 was digested with *Bsp*EI/*Stu*I to liberate an

insert of 492bp, which was substituted with the mutated 492bp fragment. This construct was sequenced utilising primers S-KR-22 and AS-KR-21. The 2083bp mutant construct was subsequently cloned into pSRαMSVtkneo using the same procedure as for pSRαMSVtkneo/*NPM-ALK*.

Δ^{MB}*NPM-ALK*: Amino acids 104 to 115 of NPM-ALK were deleted. Two PCR reactions using the 2.4kb *NPM-ALK* cDNA as template were set up in parallel using primer pairs (1) 5'END RV-1 and ΔMB-B, and (2) ΔMB-C and AS-3-ALK. The products of these reactions were used as templates for further PCR using primer pair 5'END RV-1 and AS-3-ALK. The resulting 595bp product which encompasses 36 nucleotides of 5'UT sequence and the nucleotides encoding amino acids 1-195 of *NPM-ALK*, excluding the nucleotides encoding the putative metal-binding region, was digested with *Sma*I/*Nar*I to give a 557bp fragment. This fragment was cloned into *Sma*I/*Nar*I-digested clone 20λ-1 and sequenced using the pUC M13 REVERSE, S-MB-23, pGEX-NPM and AS-ALK-24 primers. The 2017bp mutant construct was cloned into pSRαMSVtkneo using the same procedure as for pSRαMSVtkneo/*TPR-ALK*.

Δ^{64}*NPM-ALK*: Amino acids 1-64 were deleted from NPM-ALK. Utilizing the 2.4kb *NPM-ALK* cDNA as template and PCR primers S-D64-NPM and AS-3'-ALK, yielded a 423bp fragment. This PCR fragment which encompasses 30bp of 5'UT sequence and the nucleotides encoding amino acids 65-195 of

NPM-ALK was digested with *SmaI/NarI* to yield a fragment of 377bp. The 377bp fragment was cloned into *SmaI/NarI*-digested clone 20λ-1 and sequenced utilising primers pUC M13 REVERSE and AS-ALK-24. The 1867bp construct was subcloned into pSRαMSVtkneo in the same manner as for pSRαMSVtkneo/*TPR-ALK*.

$\underline{\Delta^{65\text{-}103}NPM\text{-}ALK}$: NPM amino acids 65 to 103 were deleted. In the first round of PCR, two sets of reactions were performed using the 2.1kb *XbaI/Eco*RV *NPM-ALK* cDNA insert from clone 5'-250λ-1, and primer pairs (1) 5'-Δ38 and Δ38-AS, and (2) Δ38-S and 3'-Δ38. The above PCR reactions served as templates for a second round of PCR using primers 5'-Δ38 and 3'-Δ38. The resulting 394bp product which corresponds to 52 nucleotides of 5'UT sequence and the nucleotides encoding amino acids 1-64 and 104-155 of *NPM-ALK*, was digested with *SmaI/BlpI* to yield a 340bp product. Clone 5'-250λ-1 was also digested with *SmaI/BlpI* to liberate a 457bp insert, which was replaced with the 340bp fragment. This mutant was sequenced using the pUC M13 REVERSE and AS-3'-ALK primers. The 1966bp deletion mutant was ligated into pSRαMSVtkneo as described for pSRαMSVtkneo/*TPR-ALK*.

$\underline{\Delta^{C154}NPM\text{-}ALK}$: The 154 carboxy-terminal amino acids were deleted, and a stop codon inserted. PCR was performed with the 2.4kb *NPM-ALK* cDNA template and PCR primer pair 5'-ΔC154(+) and 3'-ΔC154 to yield a 381bp product. The PCR product which corresponds to the nucleotides encoding

amino acids 412-538 of *NPM-ALK*, was digested with *Bst1107I/HindIII* to yield a 341bp product. Clone 5'-250λ-1 was also digested with *Bst1107I/HindIII* to release a 794bp insert, which was replaced with the 341bp product. The construct was sequenced utilising primers S-ΔC-24 and T7. The 1634bp mutant construct was excised with *SmaI/HindIII*, *HindIII* linkers were added and the insert was subcloned into pSRαMSVtkneo.

2.15 Antisera

Anti-ALK #11 is a rabbit polyclonal antiserum directed against amino acid residues 419-520 of NPM-ALK (Morris et al., 1996). The murine monoclonal antibody ALK-1 was also prepared using NPM-ALK residues 419-520 as an immunogen (Pulford et al., 1996). Anti-MET rabbit polyclonal Ab143 (provided by Morag Park, McGill University, Montreal, Canada) recognises amino acids 514-523 of TPR-MET, whereas the h-MET (C-28) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) is a rabbit polyclonal IgG which recognises the 28 carboxy-terminal amino acids of the human c-MET p140 β chain (Rodrigues et al., 1991). The anti-phosphotyrosine PY-20 is a mouse IgG2b monoclonal antibody; the two SHC antibodies used are a mouse monoclonal IgG1 antibody, and a rabbit affinity purified polyclonal antibody, both directed against amino acids 359-473 of the intact human

protein (Transduction Laboratories, Lexington, KY, USA). Anti-B23 is a murine polyclonal antibody directed against the entire 38kD NPM (provided by P.K. Chan, Baylor College of Medicine, Houston, TX, USA).

2.16 Immunofluorescence

COS-7 cells were electroporated with the indicated DNA constructs and grown on coverslips 24 to 36 hours prior to immunostaining. SUP-M2 and K562 cells were cytospun onto glass slides. Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed twice with PBS and permeabilised with 0.2% Triton X-100 in PBS for 10 minutes. The fixed cells were blocked in 5% dry milk in PBS for 30 minutes and subsequently incubated with either monoclonal ALK-1 (neat) or h-MET (C28) (diluted 1:500) in PBS for 30 minutes. The cells were washed six times with PBS and then incubated for 30 minutes with fluorescein (FITC)-conjugated donkey anti-rabbit or anti-mouse IgG (diluted 1:100) (Jackson Immuno-Research, West Grove, PA, USA). Following another six washes with PBS, the DNA was counterstained with 1.5µg/ml of 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI; Boehringer Mannheim, Indianapolis, IN, USA) in PBS for 5 minutes. The coverslips were mounted onto slides with Vectashield

(Vector Laboratories, Burlingame, CA, USA) and examined under an Olympus BX50 fluorescent microscope (Olympus, Japan).

2.17 *In vitro* transcription and translation

In vitro transcription and translation was performed utilising the TNT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI, USA) as per package instructions. The isotope utilised was L-[^{35}S]methionine (1000Ci/mmol; Amersham, Arlington Heights, IL, USA).

2.18 Immunoprecipitation

Cells were washed with PBS and lysed with Nonidet P-40 (NP-40) lysis buffer (25mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 44µg/ml aprotinin, 1.4µM phenylmethylsylfonyl fluoride, 2mM ethylenediamine-tetraacetic acid and 1mM sodium orthovanadate). Cell lysates were centrifuged and the supernatants were incubated with the appropriate antiserum for 2 hours at 4°C, and immune complexes were collected on protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ, USA) by incubation at 4°C for one hour. The beads were washed four times with NP-40 lysis buffer and resuspended in 2X Laemmli sample buffer (62.5mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol and a pinch of bromophenol

blue), boiled and subjected to reducing sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

2.19 Immunoblotting

Immunoprecipitates were prepared as described (see section 2.18), resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-p; Millipore, Bedford, MA, USA) using a TE70 SemiPhor Transfer Unit (Hoefer Scientific Instruments, San Fransisco, CA, USA) l . transfer buffer containing 48mM Tris, 39mM glycine, 0.037% sodium dodecyl sulphate and 20% methanol. Membranes were blocked in 3% dry milk in PBS for one hour at room temperature and incubated for a further two hours with the appropriate antibody in blocking buffer. Following three times 5 minute washes with PBS containing 0.1% Tween-20, membranes were incubated with either anti-rabbit IgG conjugated with horseradish peroxidase (1:2000) or gamma chain specific anti-mouse IgG conjugated with horseradish peroxidase (1:3000) (Zymed, S. San Fransico CA, USA)) for one hour at room temperature. Membranes were washed as stated above, and immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL, USA).

2.20 *In vitro* protein kinase assays

Immunoprecipitations were carried out as described (see section 2.18). Immune complexes were washed three times with NP-40 lysis buffer and twice with kinase buffer (25mM Hepes, pH 7.5, 6.25mM $MgCl_2$ and 12.5mM $MnCl_2$). Following the addition of 20µCi of [γ-^{32}P]ATP (6000Ci/mmol; Dupont NEN, Boston, MA, USA) in 10µl of kinase buffer containing 100µM sodium orthovanadate, samples were incubated at 37°C for 30 minutes. An equal volume of 2X Laemmli sample buffer was added and phosphorylated proteins were analysed by SDS-PAGE.

2.21 Sucrose gradient sedimentation

Stable transfectants and SUP-M2 cells were lysed in either NP-40 lysis buffer or CSK buffer (10mM Pipes, pH 7, 100mM KCl, 0.5% NP-40, 300mM sucrose and 3mM $MgCl_2$). Lysates were layered over 4.5-ml gradients of 5 to 20% sucrose in either NP-40 lysis buffer or 20mM Tris-HCl, pH 7.5, 100mM NaCl and 0.1% NP-40. Gradients were centrifuged at 4°C for 6 to 8 hours in a Beckman SW50.1 rotor at 45 000rpm. Fractions of 250µl were collected from the top of the gradient, immunoprecipitated with either anti-ALK #11 or anti-MET Ab143, and assayed *in vitro* for kinase activity or subjected to Western blotting following SDS-PAGE. Native molecular weight markers

(horse apoferritin (443kD), β-amylase (200kD), alcohol dehydrogenase (150kD), bovine serum albumin (66kD) and carbonic anhydrase (29kD) (Sigma, St. Louis, MO, USA)) were analysed in parallel gradients. These fractions were mixed with an equal volume of 2X Laemmli sample buffer, resolved by SDS-PAGE, and detected by staining the gel with Coomassie blue.

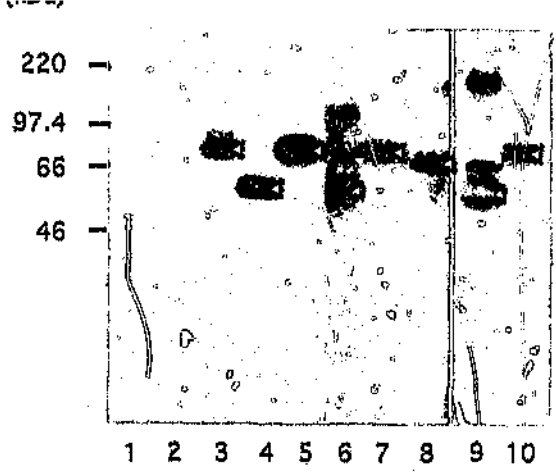
2.22 *In vivo* tumorigenicity assay

Clonal stably transfected Fr3T3 or parental cells ($5X10^6$) were washed with PBS and resuspended in 200µl PBS. The cells were injected subcutaneously into the back of NOD/LtSz-*scid* mice (Jackson Laboratories, Bar Harbor, ME, USA) (Shultz et al., 1995). Tumour formation was monitored for 10 weeks.

3. RESULTS

3.1 Transient expression of NPM-ALK, TPR-MET and mutant proteins in COS-7 cells

Following the cloning of the DNA constructs into the retroviral vector pSRαMSVtkneo (section 2.14), the constructs were tested for expression in COS-7 cells. Lysates from transfected COS-7 cells (section 2.3) were immunoprecipitated with the appropriate antibody, and expression was assessed by either *in vitro* kinase assay or by immunoblotting (sections 2.18, 2.19 and 2.20). Consistent with the calculated molecular mass, each immunoprecipitated protein migrated at the expected size: NPM-ALK, 75kDa; TPR-MET, 60-65kDa; TPR-ALK, 78kDa; NPM-MET, 55kDa; $^{M6/7/9L}$NPM-ALK, 75kDa; ^{K210R}NPM-ALK, 75kDa; Δ^{MB}NPM-ALK, 74kDa; Δ^{64}NPM-ALK, 68kDa; $\Delta^{65\text{-}103}$NPM-ALK, 71kDa and Δ^{C154}NPM-ALK, 58kDa (figure 3.1 A and B). For reasons unknown, Δ^{MB}NPM-ALK and Δ^{64}NPM-ALK did not exhibit *in vitro* kinase activity in transfected COS-7 cells, and thus the immunoprecipitates from *in vitro* transcribed and translated protein (section 2.17) were tested for *in vitro* kinase activity. Both Δ^{MB}NPM-ALK and Δ^{64}NPM-ALK expressed in this way exhibited catalytic activity *in vitro* (figure 3.1 C).



(B)

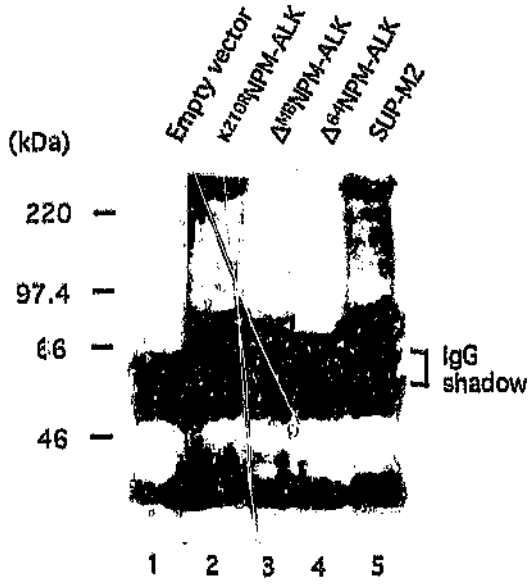


Figure 3.1. See figure legend on the following page.

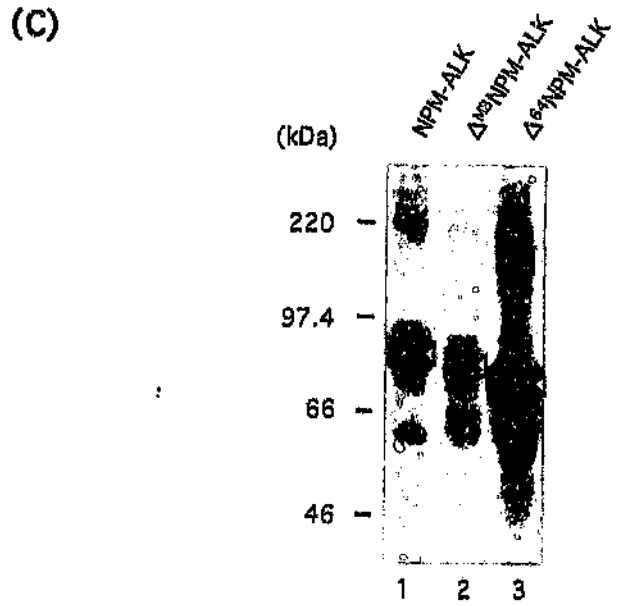


Figure 3.1. NPM-ALK, TPR-MET and engineered mutants are efficiently expressed in COS-7 cells. (A), (B) COS-7 cells were transiently transfected with the indicated DNA constructs in the retroviral vector pSRαMSVtkneo, and tested for expression. (A) Catalytic activity of NPM-ALK mutants was tested by *in vitro* kinase assays, as described in section 2.20 of Materials and Methods. Lysates from COS-7 transfected with empty vector (lane 1), *NPM-ALK*, *TPR-ALK*, $^{M5/7/9L}$*NPM-ALK*, $\Delta^{65\text{-}103}$*NPM-ALK* or Δ^{C154}*NPM-ALK*, and from the SUP-M2 cell line were immunoprecipitated using anti-ALK #11 (1:100). Lysates from COS-7 transfected with empty vector (lane 2), *TPR-MET* and *NPM-MET* were immunoprecipitated with anti-MET Ab143 (1:1000). (B) Lysates from COS-7 transfected with the indicated DNA constructs were immunoprecipitated with anti-ALK #11 and immunoblotted with ALK-1 (1:500). (C) Because the *in vitro* autokinase activity of the Δ^{MB}NPM-ALK and Δ^{64}NPM-ALK proteins expressed in COS-7 cells was difficult to demonstrate for reasons unknown, *in vitro* transcribed and translated proteins were immunoprecipitated with anti-ALK #11 and tested for *in vitro* kinase activity. All proteins were resolved by 7.5% SDS-PAGE. Each protein is indicated by an arrowhead: NPM-ALK, 75kDa; TPR-MET, 60-65kDa; TPR-ALK, 78kDa; NPM-MET, 55kDa; $^{M5/7/9L}$NPM-ALK, 75kDa; ^{K210R}NPM-ALK, 75kDa; Δ^{MB}NPM-ALK, 74kDa; Δ^{64}NPM-ALK, 68kDa; $\Delta^{65\text{-}103}$NPM-ALK, 71kDa and Δ^{C154}NPM-ALK, 58kDa. The high molecular mass bands seen in (A) lanes 3, 7, and 9, and (B) lanes 2 and 5, probably represent oligomeric complexes of the indicated protein that are too large to be resolved in these gels.

3.2 Transformation of Fr3T3 fibroblasts by NPM-ALK

To test the oncogenic potential of NPM-ALK, the entire coding sequence was cloned into the retroviral vector pSRαMSVtkneo (section 2.14) (Muller et al., 1991). Helper-free retroviral stock, prepared by transient hyperexpression in 293T cells co-transfected with this *NPM-ALK* construct together with the ψ2 packaging construct, was used to infect low-passage-number Fr3T3 fibroblasts (sections 2.3, 2.4, 2.5 and 2.6) (Pear et al., 1993). Fibroblasts infected with retroviral stock prepared using "empty" pSRαMSVtkneo as a control did not yield anchorage-independent clones in numbers greater than observed as spontaneous background with the parental Fr3T3 cells (figure 3.2 A and B). By contrast, cells infected with NPM-ALK retroviral stock formed multiple anchorage-independent clones of densely packed cells which were visible 5 to 14 days after plating in soft agar (figure 3.2 C). Fr3T3 cells infected with retroviral stock prepared using the *TPR-MET* construct were used as a positive control (Fixman et al., 1995; Rodrigues and Park, 1993) (figure 3.2 D). Anchorage-independent colonies were photographed at a magnification of 10X to illustrate the morphology of the individual clones (figure 3.3 A-D).

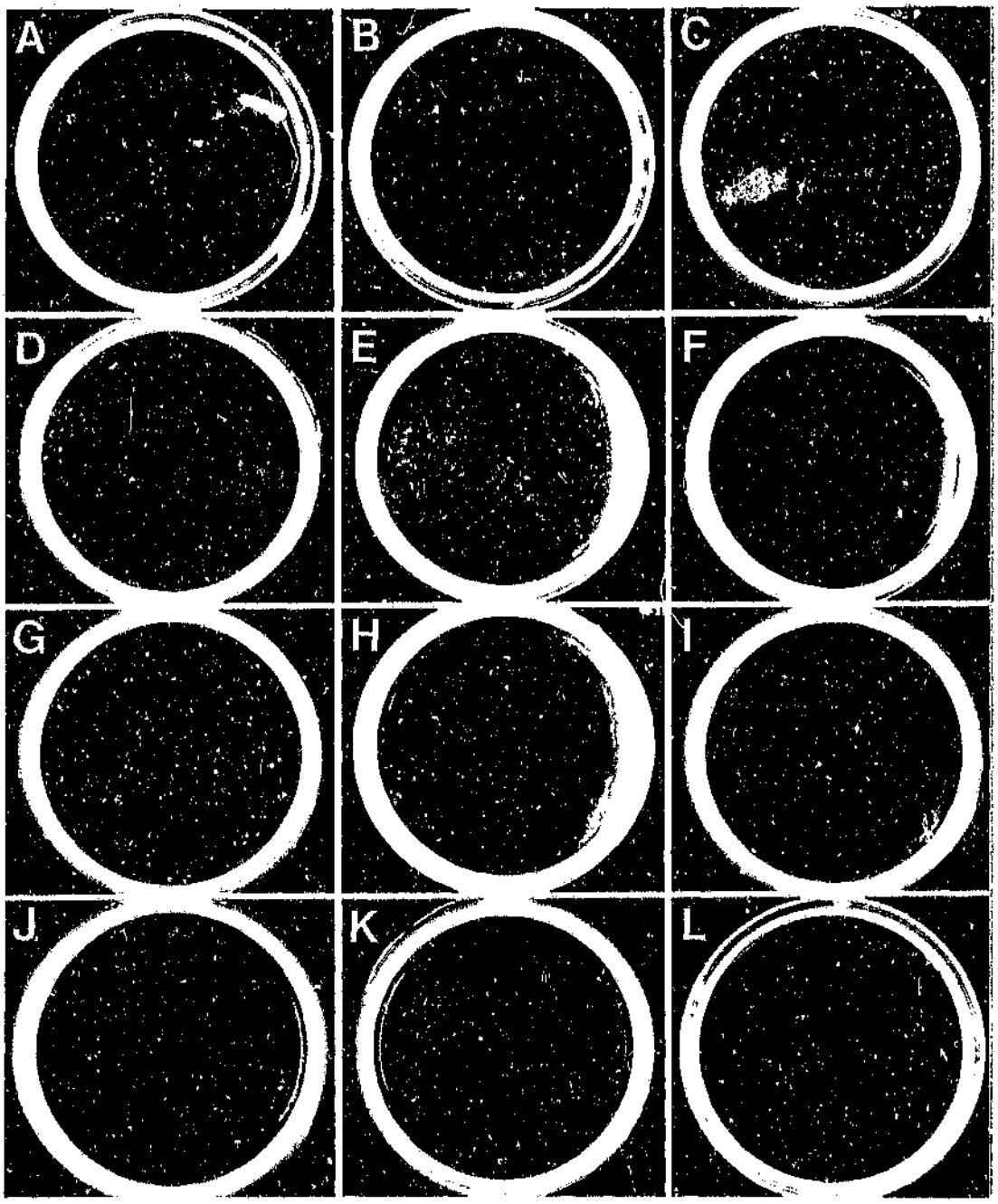


Figure 3.2. Anchorage-independent growth of Fr3T3 cells expressing the indicated protein. Equivalent numbers of cells ($5X10^3$) were seeded in soft agar 72 hours after infection. (A) Uninfected parental Fr3T3, (B) "empty" pSRαMSVtkneo retroviral vector, (C) NPM-ALK, (D) TPR-MET, (E) TPR-ALK, (F) NPM-MET, (G) $^{M5/7/9L}$NPM-ALK, (H) ^{K210R}NPM-ALK, (I) Δ^{MB}NPM-ALK, (J) Δ^{84}NPM-ALK, (K) $\Delta^{65\text{-}103}$NPM-ALK, (L) Δ^{C154}NPM-ALK. The soft agar plates were photographed after two weeks in culture.

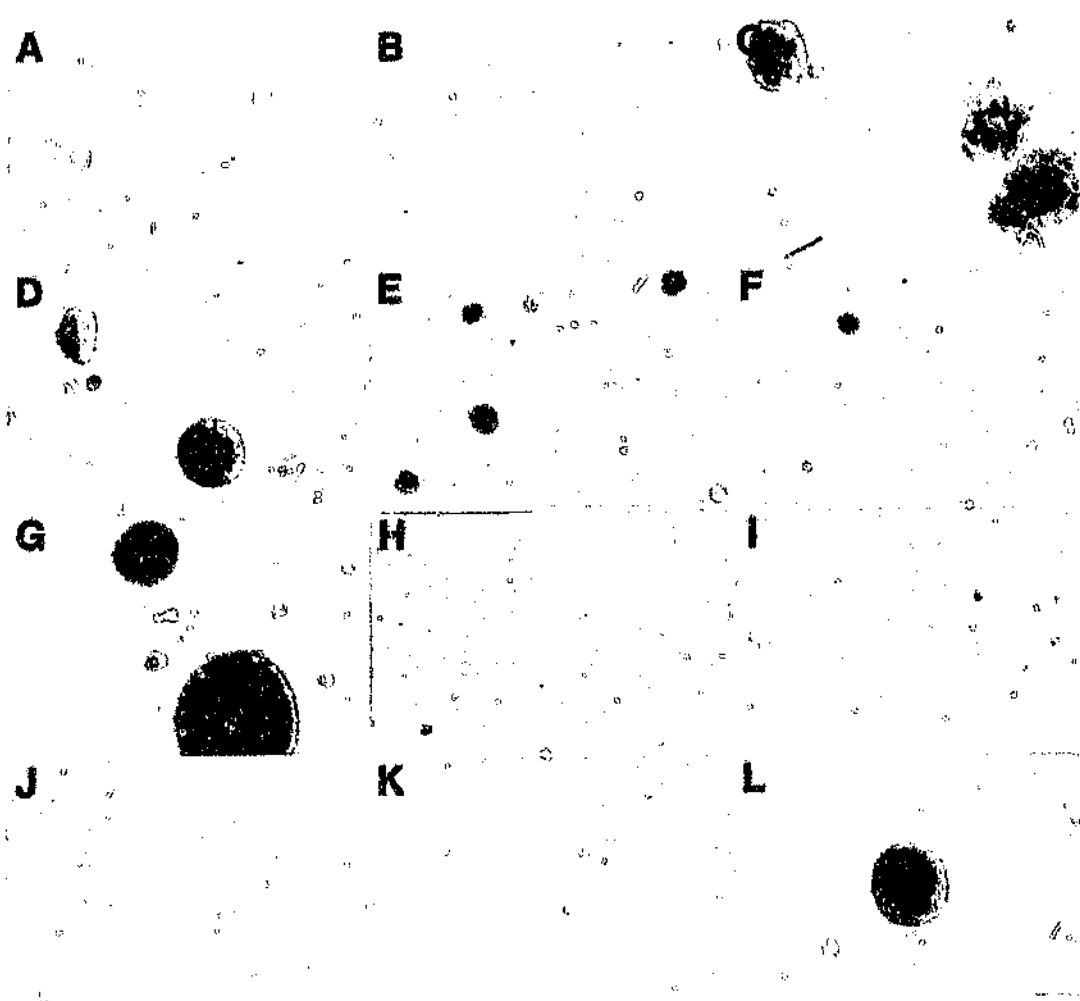


Figure 3.3. Anchorage-independent growth of Fr3T3 cells expressing the indicated protein (magnified). The soft agar cloning dishes in figure 3.2 were photographed at a 10X magnification to show individual clones. (A) Uninfected parental Fr3T3, (B) "empty" pSRαMSVtkneo retroviral vector, (C) NPM-ALK, (D) TPR-MET, (E) TPR-ALK, (F) NPM-MET, (G) $^{M5/7/9L}$NPM-ALK, (H) ^{K210R}NPM-ALK, (I) Δ^{MB}NPM-ALK, (J) Δ^{64}NPM-ALK, (K) $\Delta^{68\text{-}103}$NPM-ALK, (L) Δ^{C154}NPM-ALK.

Further evidence of NPM-ALK-induced transformation was provided by focus formation assays (section 2.7). Infected Fr3T3 cells were cultured in 100mm tissue culture dishes for 10 to 14 days and then stained with Giemsa. Fr3T3 cells infected with NPM-ALK retroviral stock formed numerous foci (figure 3.4 C). Fibroblasts infected with viral stock prepared using *TPR-MET* served as a positive control (figure 3.4 D). Fr3T3 cells infected with retrovirus using "empty" retroviral vector exhibited a low background of focus formation which was comparable to that of parental Fr3T3 cells (figure 3.4 A and B). Fibroblasts infected with NPM-ALK viral stock were densely packed, morphologically transformed cells that coalesced upon further growth to form sheets of abnormal cells, whereas parental cells and those expressing "empty" vector exhibited a normal untransformed morphology (figure 3.5 A-D).

In vivo tumorigenicity of one Fr3T3 clone expressing high levels of NPM-ALK (designated NPM-ALK clone #1) was assessed by subcutaneous injection of $5X10^6$ cells into the back of each NOD/LtSz-*scid* mouse (section 2.22). Tumours with an average diameter of approximately 2cm formed in 5 to 14 days at the site of injection (figure 3.6 A and B). No tumours formed in the mice injected with either parental Fr3T3 cells or Fr3T3 cells expressing "empty" pSRαMSVtknec, even after 10 weeks.

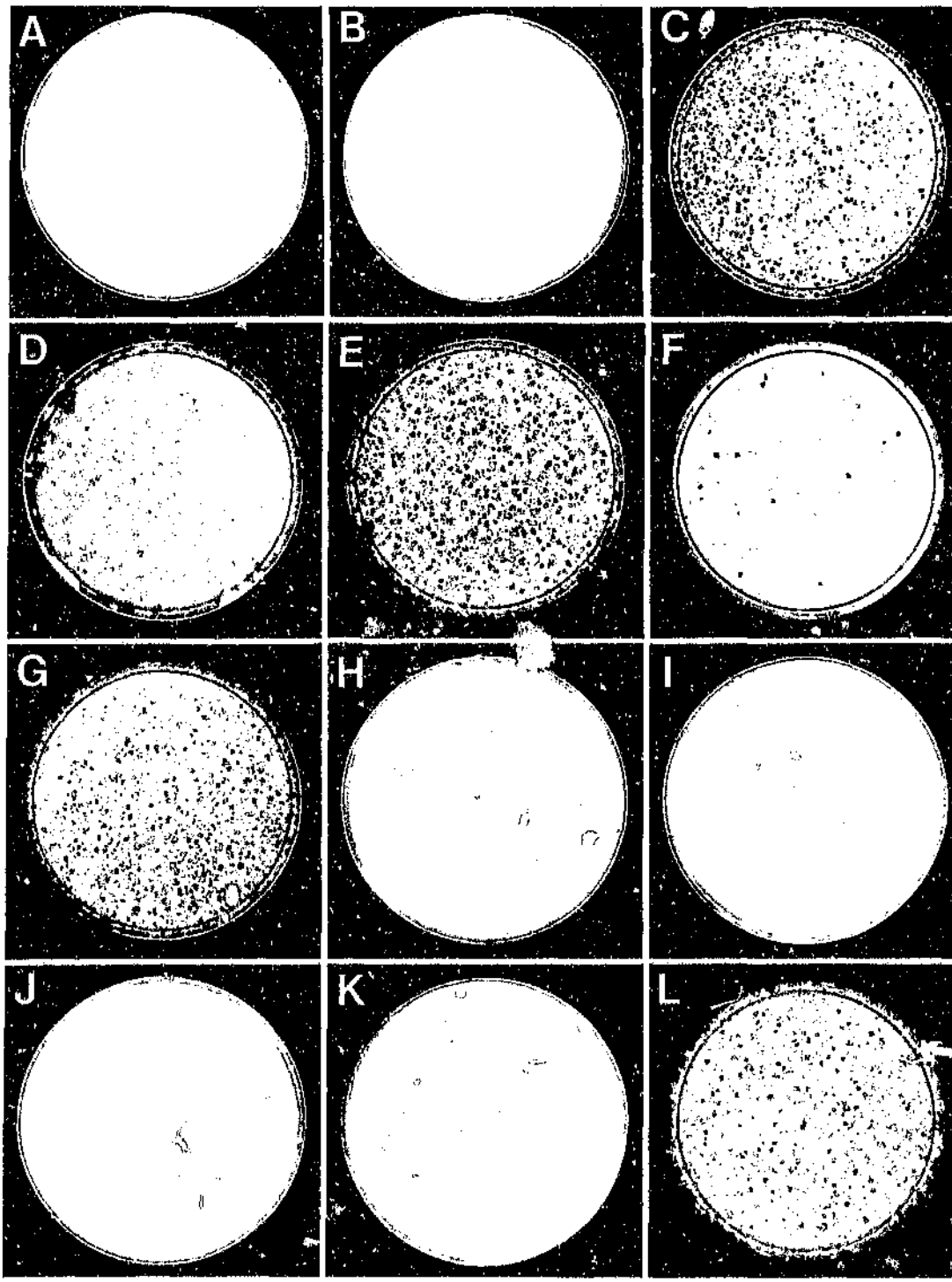


Figure 3.4. See figure legend on the following page.

Figure 3.4. Focus formation of NPM-ALK, TPR-MET and engineered mutant proteins. Fr3T3 fibroblasts were infected with retroviral stock prepared using the indicated construct, seeded into 100mm tissue culture dishes, then stained with Giemsa after 10 to 14 days in culture. (A) Uninfected parental Fr3T3, (B) "empty" pSRαMSVtkneo retroviral vector, (C) NPM-ALK, (D) TPR-MET, (E) TPR-ALK, (F) NPM-MET, (G) $^{M5/7/9L}$NPM-ALK, (H) ^{K210R}NPM-ALK, (I) Δ^{M3}NPM-ALK, (J) Δ^{64}NPM-ALK, (K) $\Delta^{85\text{-}103}$NPM-ALK, (L) Δ^{C154}NPM-ALK.

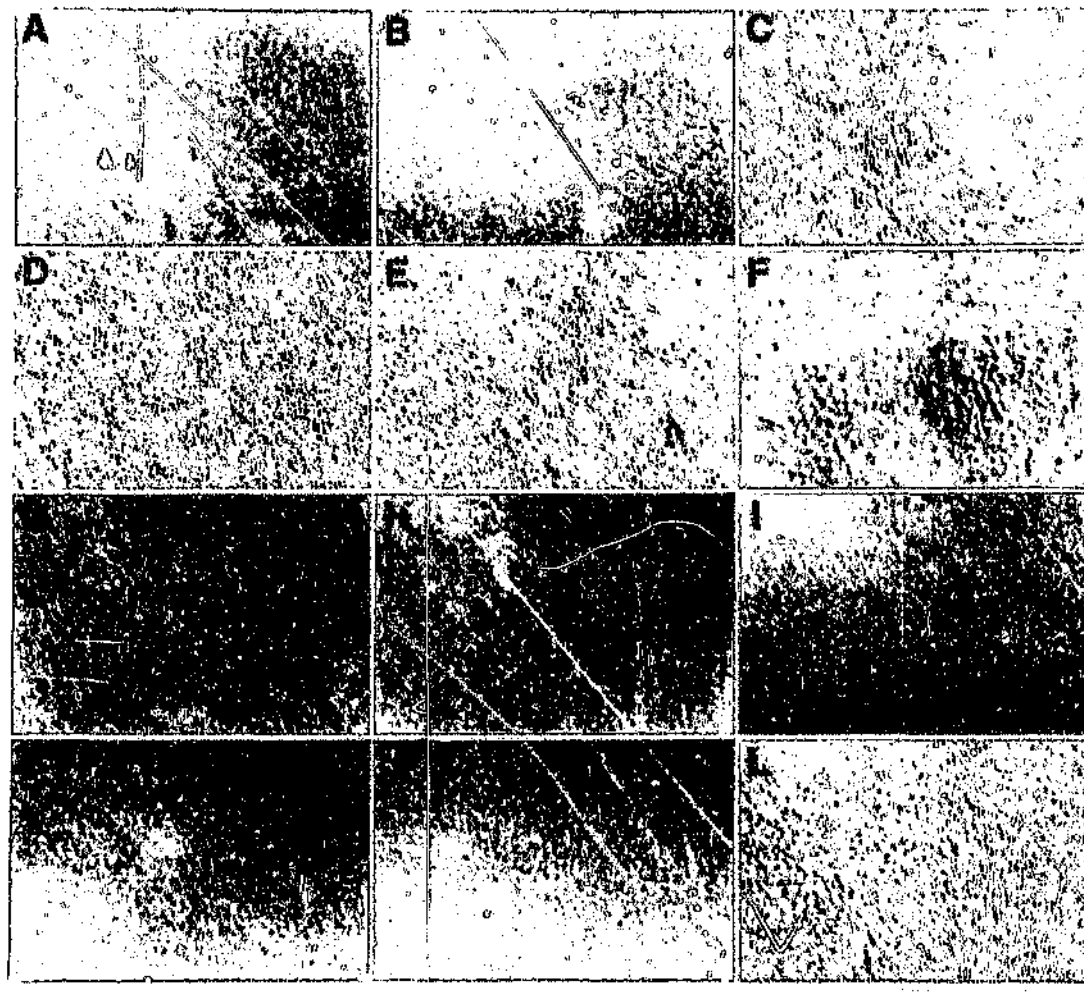
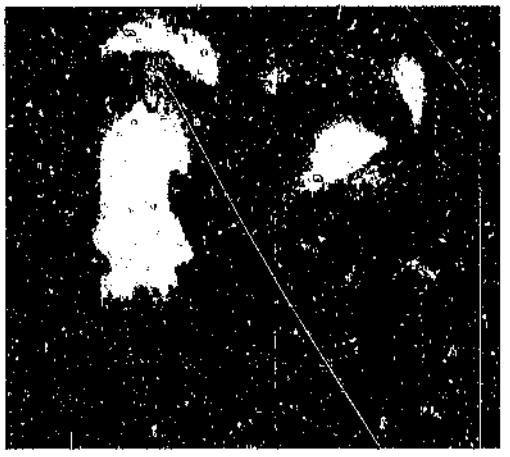


Figure 3.5. Morphology of Fr3T3 cells expressing NPM-ALK, TPR-MET and engineered proteins. (A) Uninfected parental Fr3T3, (B) "empty" pSRαMSVtkneo retroviral vector, (C) NPM-ALK, (D) TPR-MET, (E) TPR-ALK, (F) NPM-MET, (G) $^{M5/7/9L}$NPM-ALK, (H) ^{K210R}NPM-ALK, (I) Δ^{MB}NPM-ALK, (J) Δ^{64}NPM-ALK, (K) $\Delta^{95\text{-}103}$NPM-ALK, (L) Δ^{C154}NPM-ALK.

(A)



(B)

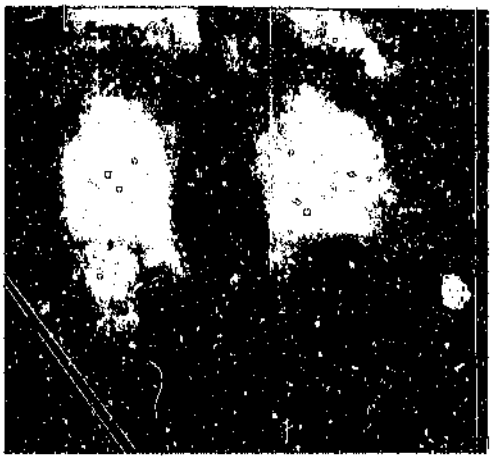


Figure 3.6. *in vivo* tumorigenicity of NPM-ALK. NOD/LtSz-*scid* mice were subcutaneously injected with $5X10^6$ clonally-derived parental Fr3T3 cells or clonal fibroblasts expressing either "empty" pSRαMSVtkneo or NPM-ALK. Tumours with an average diameter of approximately 2cm formed in 5 to 14 days at the site of injection in both the male and female mice. (A) Female mice injected with either parental Fr3T3 cells or clonal Fr3T3 cells expressing NPM-ALK. (B) Male mice injected with clonal Fr3T3 cells expressing either "empty" vector or NPM-ALK.

(A)

(B)

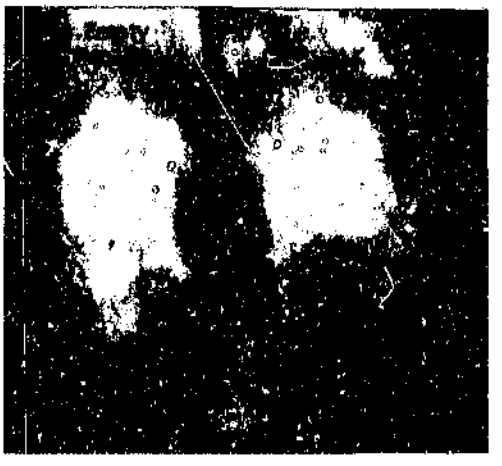


Figure 3.6. *In vivo* tumorigenicity of NPM-ALK. NOD/LtSz-*scid* mice were subcutaneously injected with $5X10^6$ clonally-derived parental Fr3T3 cells or clonal fibroblasts expressing either "empty" pSRαMSVtkneo or NPM-ALK. Tumours with an average diameter of approximately 2cm formed in 5 to 14 days at the site of injection in both the male and female mice. (A) Female mice injected with either parental Fr3T3 cells or clonal Fr3T3 cells expressing NPM-ALK. (B) Male mice injected with clonal Fr3T3 cells expressing either "empty" vector or NPM-ALK.

3.3 Transformation of fibroblasts by NPM-ALK requires the kinase activity of the truncated ALK protein

The characterisation of other truncated, fused receptor tyrosine kinases such as TPM3-TRK and TPR-MET has indicated that their oncogenicity is dependent upon the ability of the respective kinase to phosphorylate cellular substrates, some of which relay mitogenic signals to the cell nucleus (Rodrigues and Park, 1994). To confirm that the intrinsic kinase activity of ALK is required for NPM-ALK-mediated transformation, a kinase-defective mutant, ^{K210R}NPM-ALK, was prepared in which the invariant lysine residue in the ATP-binding region of the catalytic domain of protein kinases was changed to an arginine (section 2.14) (Hanks et al., 1988). As expected, the ^{K210R}NPM-ALK mutant failed to transform fibroblasts (figures 3.2 H, 3.3 H, 3.4 H and 3.5 H).

Another mutant, Δ^{C154}NPM-ALK, was analysed in which the ALK portion of the chimaera was altered by deletion of the carboxy-terminal 154 amino acids (section 2.14). This mutant was prepared to determine if these carboxy-terminal residues, which include the potential SHC and IRS-1 binding site NPTY^{867}, are required for transformation (van der Geer and Pawson, 1995). Fr3T3 fibroblasts were infected with retroviral stock prepared using Δ^{C154}*NPM-ALK*. Deletion of this large portion of the carboxy-terminal tail of

ALK only slightly reduced the transforming activity as compared to wild-type NPM-ALK (figures 3.2 L, 3.3 L, 3.4 L, 3,5 L and 3.7). Lysates from SUP-M2 cells or from 293T cells transfected with Δ^{C154}NPM-ALK were immunoprecipitated using either anti-SHC or anti-ALK antibodies (section 2.3 and 2.18). These results revealed that wild-type NPM-ALK associated physically with SHC, whereas Δ^{C154}NPM-ALK failed to do so (figure 3.8), indicating that NPTY^{667} is a SHC binding site.

3.4 An intact NPM segment is required for transformation by NPM-ALK

The absence of any sequence differences between the portion of ALK present in NPM-ALK and the corresponding region of the full-length normal receptor suggested that the NPM residues of the chimaera function to activate its transforming ability (Morris et al., 1994; Morris et al., 1996). To examine this possibility, three deletion mutants of NPM-ALK were prepared in which non-overlapping portions of the 118 amino acid NPM were removed: Δ^{64}NPM-ALK, in which the amino-terminal 64 residues are deleted; $\Delta^{65\text{-}103}$NPM-ALK, in which amino acids 65 to 103 are absent; and Δ^{MB}NPM-ALK, in which residues 104 to 115 that comprise a C-X_5-H-X_4-H motif of unknown function and referred to as a putative metal-binding domain are removed (section 2.14) (Chan et al., 1989) (figure 3.7). Each of these NPM deletion mutants

	No. foci	Oligomerisation	Subcellular Localisation	
			Cytoplasmic	Nuclear
NPM-ALK	1310	Y	Y	Y
TPR-MET	2560	Y	Y	N
TPR-ALK	950	Y	Y	N
NPM-MET	480	Y	Y	Y
NPM-ALK	1280	Y	Y	Y
NPM-ALK	0	ND	Y	Y
NPM-ALK	0	N	Y	N
NPM-ALK	0	N	Y	N
NPM-ALK	0	N	Y	N
NPM-ALK	970	ND	Y	Y

Figure 3.7. See figure legend on the following page.

Figure 3.7. Schematic of NPM-ALK mutants. Diagrammatic representation of wild-type NPM-ALK, the NPM-ALK mutant proteins analysed in this study, and the wild-type TPR-MET protein are illustrated (Rodrigues and Park, 1993). Indicated motifs include the putative metal-binding (MB) domain on NPM-ALK and the leucine zipper (LZ) regions of TPR-MET. The transforming capability (as indicated by the average number of foci per 100mm dish), ability to oligomerise, and subcellular location of each protein are indicated to the right.

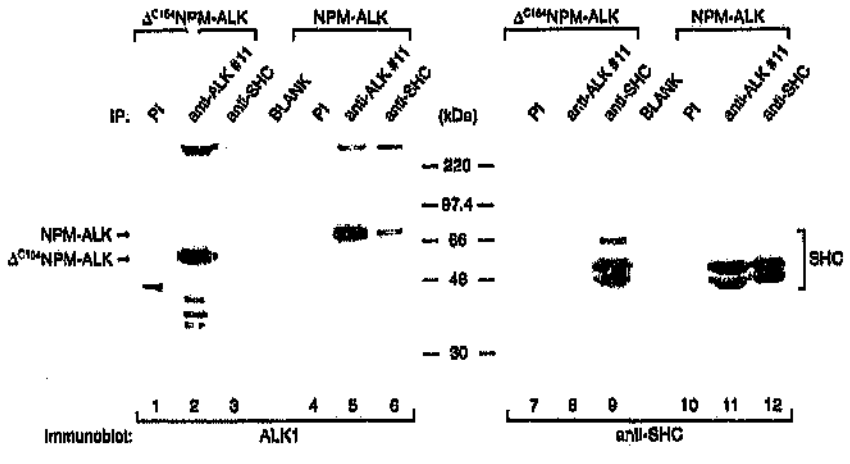


Figure 3.8. Association of NPM-ALK and Δ^{C154}NPM-ALK with SHC. Lysates from 293T cells transfected with Δ^{C154}NPM-ALK, or from SUP-M2 cells, which express wild-type NPM-ALK, were immunoprecipitated with either anti-ALK #11 (1:100) or polyclonal anti-SHC (1:80). To examine co-immunoprecipitation of SHC with either NPM-ALK and/or Δ^{C154}NPM-ALK, PVDF membranes were immunoblotted, as indicated, with either ALK-1 (1:50) or monoclonal anti-SHC (1:250). IP: immunoprecipitation; PI: pre-immune.

failed to transform Fr3T3 fibroblasts as judged by either soft agar colony formation (figures 3.2 I-K and 3.3 I-K) or focus formation (figures 3.4 I-K and 3.5 I-K). Importantly, although all of the three mutants could be demonstrated to possess autokinase activity *in vitro* (figure 3.1 A and C), none exhibited kinase activity *in vivo* (figure 3.9).

3.5 Stable expression of NPM-ALK, TPR-MET and engineered mutants in Fr3T3 cells

A number of clonal cell lines were derived from each of the infected Fr3T3 fibroblast lines (section 2.7). Infected Fr3T3 were either selected in G418-containing growth medium and individual clones were isolated utilising cloning rings, or anchorage-independent clones were picked from soft agar. These cell lines were tested for expression by either *in vitro* kinase assay or by immunoblotting (section 2.19 and 2.20). Each protein was efficiently expressed in the Fr3T3 cells (figure 3.10 A and B).

3.6 NPM-ALK exists *in vivo* as an oligomeric complex

Ligand-mediated activation of the catalytic function of receptor tyrosine kinases is thought to be generated by receptor dimerisation, resulting in intermolecular cross-phosphorylation and the ability to phosphorylate

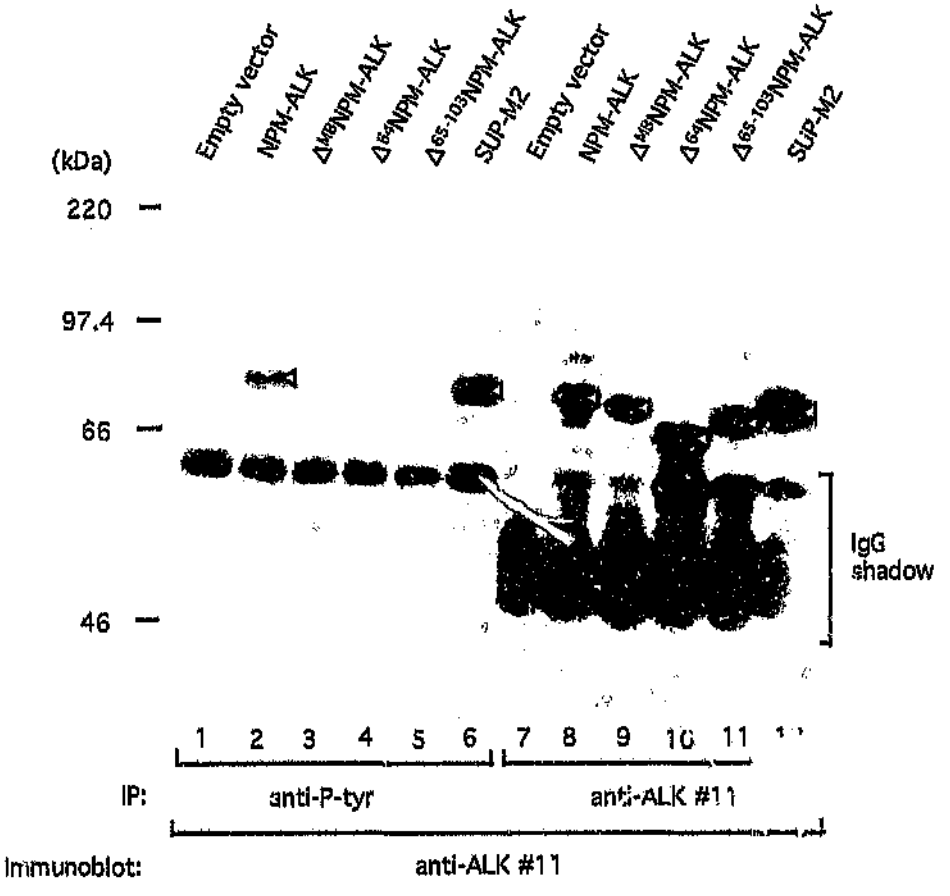


Figure 3.9. *In vivo* tyrosine phosphorylation of NPM-ALK and selected NPM-ALK mutants. Lysates from clonal Fr3T3 cells expressing the indicated proteins, and from SUP-M2 cells were immunoprecipitated using either anti-ALK #11 (1:100) or anti-phosphotyrosine (anti-P-Tyr) (1:250). After transfer to PVDF membranes, proteins were immunoblotted with anti-ALK #11 (1:500).

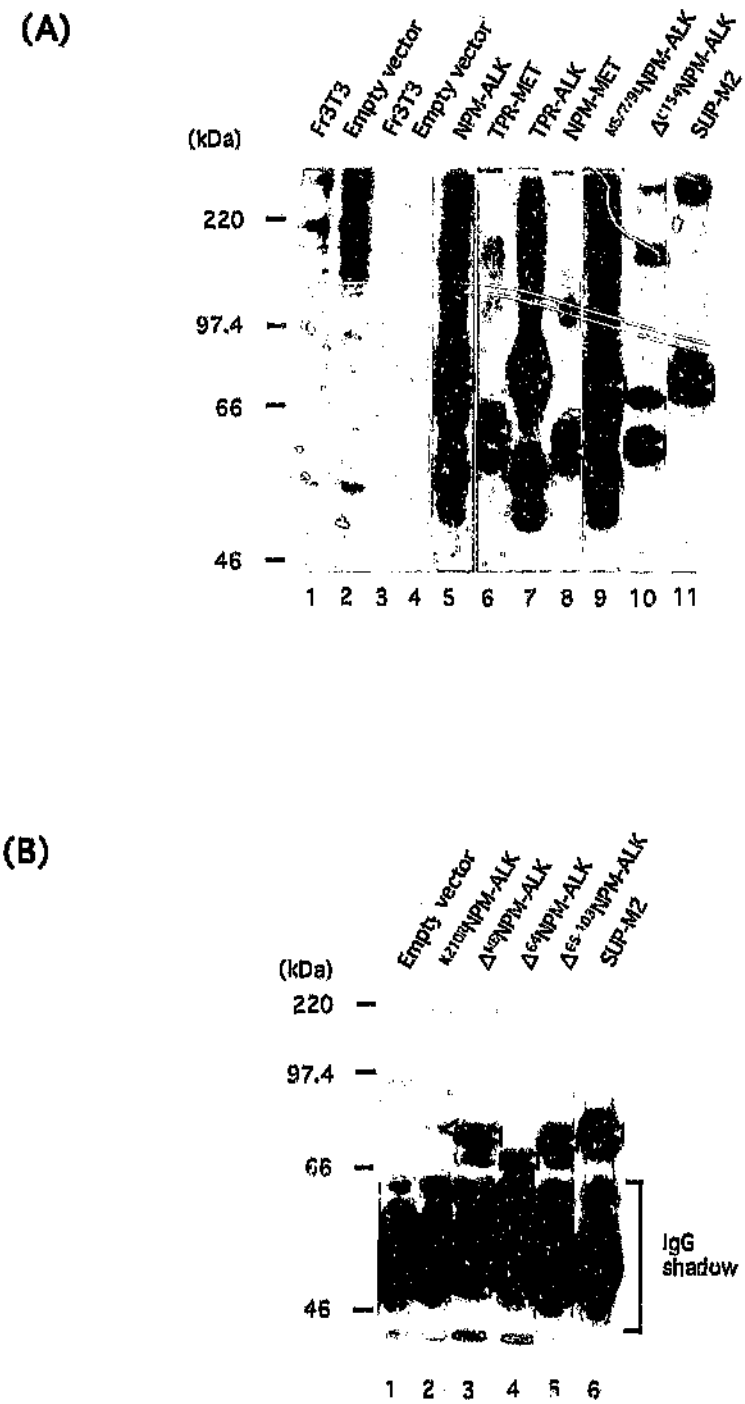


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Figure 3.10. Representative clonal Fr3T3 cell lines showing the expression of NPM-ALK, TPR-MET and mutants. (A) The catalytic activity of NPM-ALK mutants was tested by *in vitro* kinase assays. Lysates from parental Fr3T3 cells (lane 1) and from those cells expressing empty vector (lane 2), NPM-ALK, TPR-ALK, $^{M5/7/9L}$NPM-ALK, and Δ^{C154}NPM-ALK were immunoprecipitated using anti-ALK #11 (1:100). Lysates from parental Fr3T3 (lane 3) and those expressing empty vector (lane 4), TPR-MET and NPM-MET were immunoprecipitated with anti-MET Ab143 (1:1000). (B) Lysates from Fr3T3 cells expressing the indicated protein were sequentially immunoprecipitated and immunoblotted with anti-ALK #11 (1:500).

cellular substrates (Ullrich and Schlecsinger, 1990). The fact that the NPM-ALK oncogene product has constitutive kinase activity *in vivo* (figure 3.9) suggests that the chimaera has the ability to form dimers or higher-order oligomers independent of ligand stimulation.

To determine if ligand-independent dimerisation of NPM-ALK occurs, the formation of NPM-ALK complexes *in vivo* was examined. SUP-M2 cell lysates were layered on 5 to 20% sucrose gradients, and the sedimentation profile NPM-ALK was determined after centrifugation. Fractions collected from the gradients were assayed for the presence of NPM-ALK proteins by *in vitro* kinase assay (sections 2.20 and 2.21). NPM-ALK-associated kinase activity was found distributed over several gradient fractions, with a peak slightly greater than 200kDa (figure 3.11 A) - larger than the approximately 150kDa size predicted for NPM-ALK homodimers. In addition to forming homo-oligomers *in vitro* (figure 3.11 C) and *in vivo* (figure 3.11 A), NPM-ALK was found to form hetero-oligomers with normal NPM *in vivo* (figure 3.11 D).

To determine if the loss of transforming ability and *in vivo* kinase activity of the NPM deletion mutants Δ^{84}NPM-ALK, $\Delta^{65\text{-}103}$NPM-ALK, and Δ^{MB}NPM-ALK correlated with the inability of these proteins to oligomerise, similar sucrose

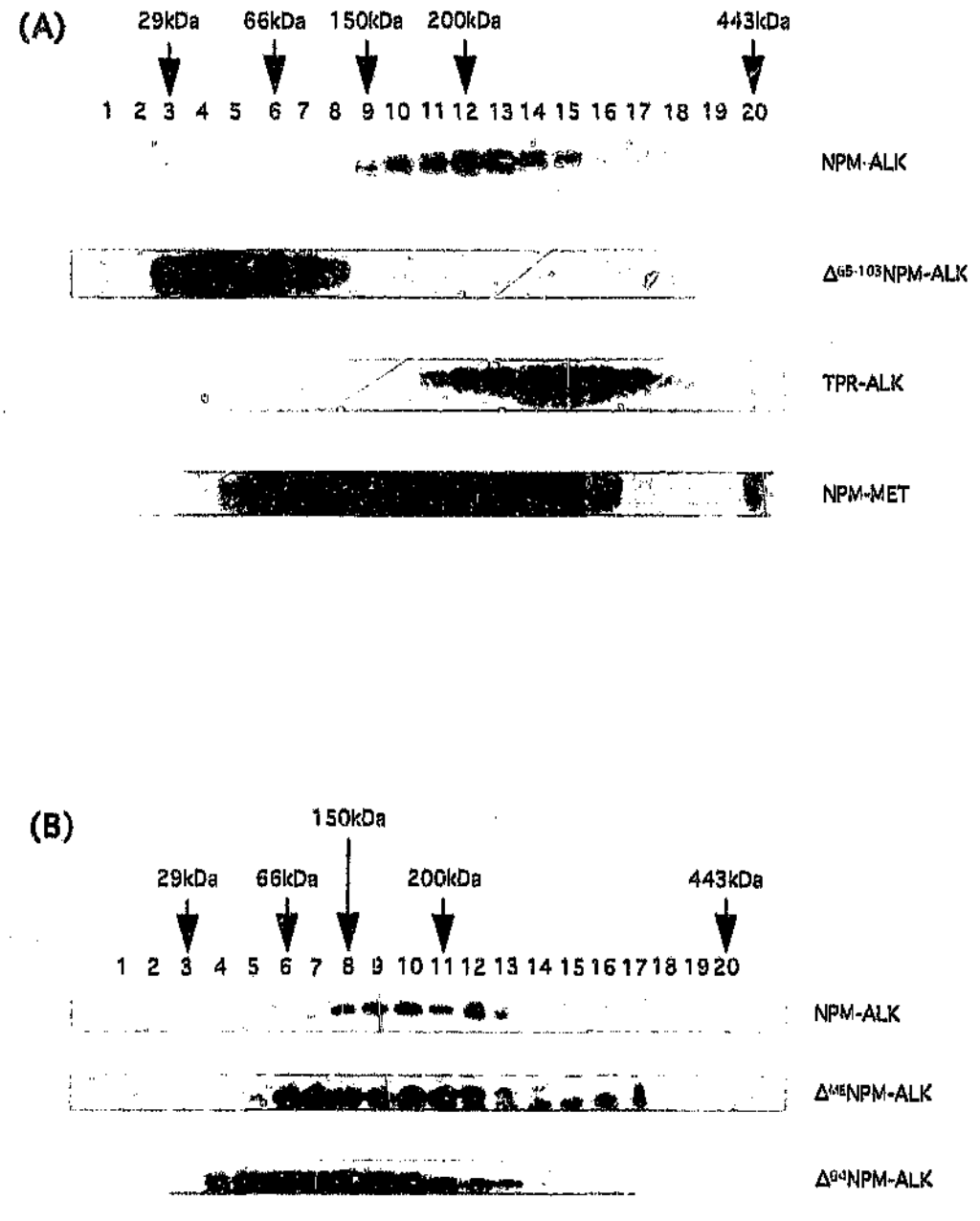

Figure 3.11. See figure legend on page 89.

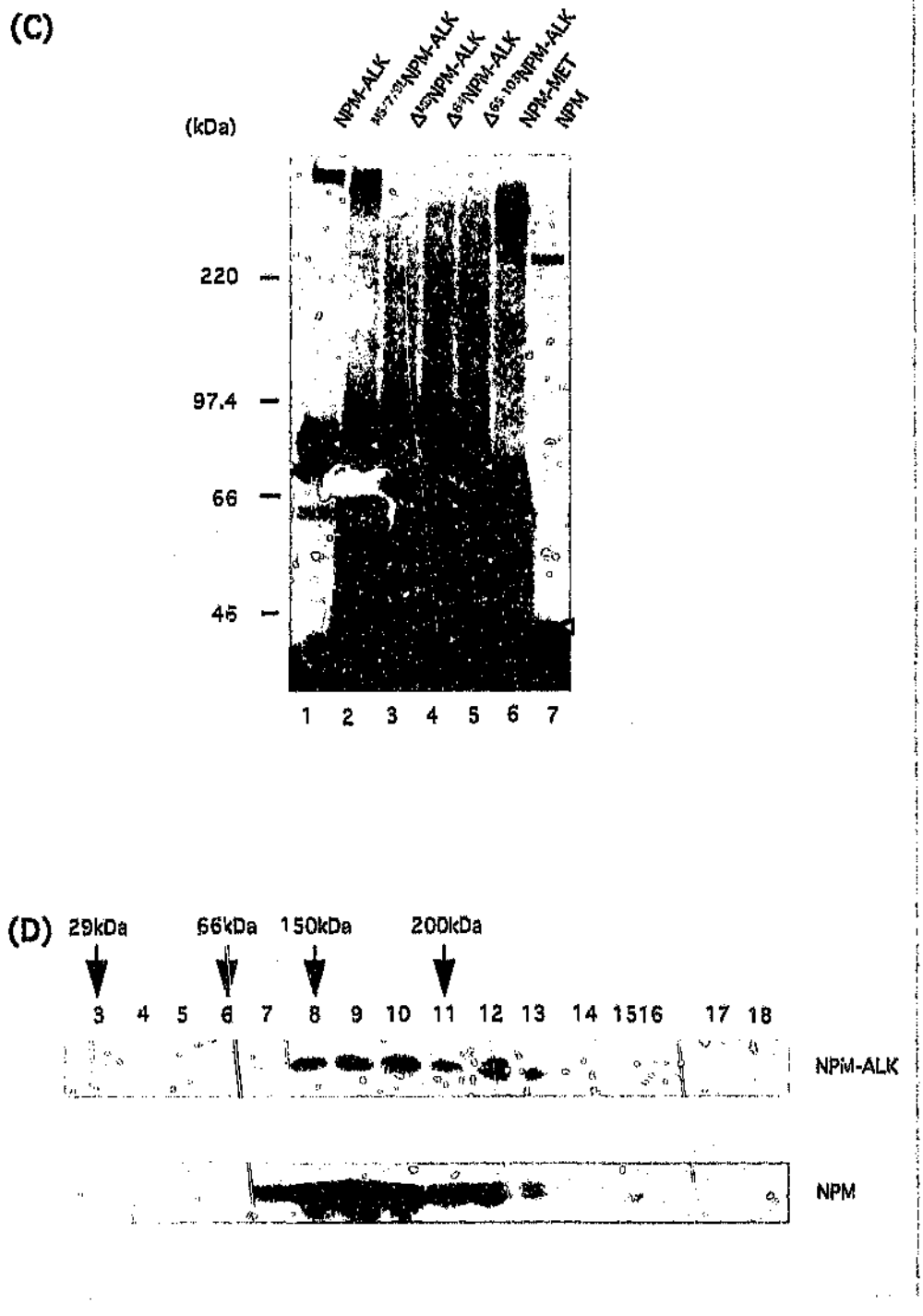

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Figure 3.11. Oligomerization of NPM-ALK and selected NPM-ALK mutants. (A), (B) Cells from the t(2;5)-positive lymphoma cell line SUP-M2 (for NPM-ALK) or clonal Fr3T3 cells expressing either $\Delta^{65\text{-}103}$NPM-ALK, NPM-MET, Δ^{MB}NPM-ALK or Δ^{64}NPM-ALK were lysed in NP-40 lysis buffer, while Fr3T3 cells expressing TPR-ALK were lysed in CSK buffer. Lysates from SUP-M2 cells and from Fr3T3 cells expressing either $\Delta^{65\text{-}103}$NPM-ALK, NPM-MET, Δ^{MB}NPM-ALK or Δ^{64}NPM-ALK were layered over 5 to 20% sucrose gradients containing NP-40 lysis buffer, whereas lysates from Fr3T3 cells expressing TPR-ALK were layered over a 5 to 20% gradient containing 20mM Tris, pH 7.5, 100mM NaCl and 0.1% NP-40. The number above each lane corresponds to the fraction number from the lightest fraction to the heaviest. Proteins were separated by SDS-PAGE under reducing conditions. Fractions were immunoprecipitated using either anti-ALK #11 (1:100) (for NPM-ALK, $\Delta^{65\text{-}103}$NPM-ALK, TPR-ALK, Δ^{MB}NPM-ALK and Δ^{64}NPM-ALK) or anti-MET Ab143 (1:1000) (for NPM-MET) and then assayed for activity by *in vitro* kinase assay (A) or by Western blotting (B). The monomeric forms of NPM-ALK (75kDa), $\Delta^{65\text{-}103}$NPM-ALK (71kDa), TPR-ALK (78kDa), NPM-MET (55kDa), Δ^{MB}NPM-ALK (74kDa) and Δ^{64}NPM-ALK (68kDa) are shown. (C) NPM-ALK, the indicated mutants of NPM-ALK and NPM were transcribed and translated *in vitro*. Unboiled samples were resolved by 6% SDS-PAGE under reducing conditions, as previously described (Liu and Chan, 1991). The monomeric form of each protein is shown by an arrowhead: NPM-ALK, 75kDa; $^{M6/7/0L}$NPM-ALK, 75kDa; Δ^{MB}NPM-ALK, 74kDa; Δ^{64}NPM-ALK, 68kDa; $\Delta^{65\text{-}103}$NPM-ALK, 71kDa; NPM-MET, 55kDa; and NPM, 38kDa. (D) NPM-ALK is physically associated with normal NPM in t(2;5)-positive lymphoma cells. Gradient fractions of SUP-M2 lysates were immunoprecipitated with anti-ALK #11 and immunoblotted with either anti-ALK #11 or anti-B23.

gradient fractionations using lysates from Fr3T3 cells stably expressing each mutant were performed. Although each of the 3 proteins distributed within the gradients at somewhat different relative molecular masses, all three sedimented with a peak significantly smaller than that of the wild-type chimaeric protein (figure 3.11 A and B). The results obtained from the sucrose gradient fractionations for Δ^{MB}NPM-ALK and Δ^{64}NPM-ALK were inconclusive, and as such further experiments were performed. To determine whether or not the NPM deletion mutants could homo-oligomerise, *in vitro* transcribed and translated proteins that had not been boiled were resolved by SDS-PAGE (figure 3.11 C). Whereas NPM-ALK was able to homo-oligomerise *in vitro*, none of the NPM deletion mutants were able to do so, indicating that complex formation was indeed inhibited by these alterations of the NPM moiety. Furthermore, Δ^{MB}NPM-ALK was unable to associate with normal NPM (data not shown). That complex formation is mediated by the NPM portion of the fusion is consistent with data indicating that normal NPM exists *in vivo* as a homo-oligomer (Liu and Chan, 1991; Yung and Chan, 1987).

Structure-function study of normal NPM has suggested that, within the amino-terminal region that mediates homo-oligomerisation, methionine residues at positions 5, 7 and 9 are essential for oligomer formation (Liu

and Chan, 1991). Therefore an engineered NPM-ALK mutant protein, $^{M3/7/8L}$NPM-ALK, in which these residues were changed to leucines, recapitulating mutations reported to inhibit normal NPM hexamer formation. Interestingly, $^{M6/7/8L}$NPM-ALK sedimented in sucrose gradients as a complex identical in mass to the wild-type NPM-ALK (data not shown), and was able to homo-oligomerise *in vitro* in a similar fashion to NPM-ALK (figure 3.11 C). This mutant protein was efficiently phosphorylated *in vivo* (data not shown), and transformed Fr3T3 with the same potency as the wild-type chimaera (figures 3.2 G, 3.3 G, 3.4 G, 3.5 G and 3.7). These results suggest that the exact residues required for NPM homo-oligomerisation versus NPM and NPM-ALK heterodimerisation may differ somewhat.

Although providing evidence that the NPM portion of NPM-ALK serves as an oligomerisation motif, the above experiments do not rule out the possibility that this segment could perform other functions critical for transformation. To better address this issue an ALK mutant protein , TPR-ALK, was prepared, in which NPM was replaced with the residues of the TPR protein that are present in the TPR-MET oncogene (section 2.14) (Park et al., 1986). This portion of TPR, a nuclear pore protein, contains two leucine zipper motifs that have been shown to mediate dimerisation of TPR-MET and produce its constitutive kinase activation (Byrd et al., 1994). No other

functions in TPR-MET have been demonstrated for these TPR activating sequences. The activation of ALK transforming activity by a fusion partner with no homology to NPM, such as TPR, would support a singular function for NPM in NPM-ALK oligomerisation. Determination of the oligomerisation state of TPR-ALK isolated from stably-expressing Fr3T3 using sucrose gradients showed the protein to exist *in vivo* as a multimeric complex greater than 200kDa (figure 3.11 A). Transformation of Fr3T3 fibroblasts by TPR-ALK was efficient, being only slightly less potent than NPM-ALK itself (figures 3.2 E, 3.3 E, 3.4 E, 3.5 E and 3.7). The reciprocal mutant, NPM-MET, was generated to ascertain whether NPM sequences could activate a heterologous truncated receptor tyrosine kinase (section 2.14). In addition to being autophosphorylated *in vivo* (data not shown) and sedimenting in sucrose gradients as a complex of roughly 100-200kDa (figure 3.11 A), NPM-MET was shown to homo-oligomerise *in vitro* (figure 3.11 C). Furthermore, this NPM-MET mutant protein transformed Fr3T3, although with only approximately one-fifth the efficiency of TPR-MET (figures 3.2 F, 3.3 F, 3.4 F and 3.5 F and 3.7). The reason(s) for the low transforming ability of NPM-MET in comparison with either NPM-ALK or TPR-MET are unclear. TPR-MET is localised to the cytoplasm, whereas NPM-MET is found in the nucleus (figure 3.12 E and 3.12 G). The unusual location of this MET fusion protein may alter its ability to transfrom fibroblasts in that the levels of NPM-MET found in the cytoplasm may be just below that required for transformation. An alternate explanation would be that NPM-MET is expressed at lower levels than TPR-MET and NPM-ALK. however, this is improbable (see figure 3.10 A).

3.7 Transformation by NPM-ALK does not require localisation to the nuclear compartment

Cell fractionation (performed by S.W. Morris) and immunofluorescent staining (section 2.16) demonstrated that the NPM-ALK in the t(2;5)-positive cell line SUP-M2, was found in both the cytoplasm and the nucleus (figure 3.12 A). K562, a t(2;5)-negative cell line, was used as a negative control (figure 3.12 B). The normal shuttling function of NPM together with the observation that NPM and NPM-ALK heterodimerise, suggests that the nuclear location of the chimaera results from NPM-mediated protein translocation since the nuclear localisation signals of NPM are not present in NPM-ALK (Borer et al., 1989; Chan et al., 1989; Dumbar et al., 1989; Schmidt-Zachmann and Franke, 1988; Schmidt-Zachmann et al., 1987). Therefore, if this hypothesis is correct, the mutant proteins Δ^{64}NPM-ALK, Δ^{68-103}NPM-ALK, and Δ^{M8}NPM-ALK that fail to heterodimerise with NPM would be predicted to be completely cytoplasmic. To determine the subcellular location of these proteins immunostaining experiments were performed with the monoclonal anti-ALK-1 antibody using COS-7 cells transiently transfected with each mutant construct (Pulford et al., 1996). Simultaneous DAPI staining was performed to clearly identify cell nuclei. None of these three mutant NPM-ALK proteins were present within the cell nucleus (figure 3.12 G-I). To provide additional evidence that shuttling by NPM can produce nuclear

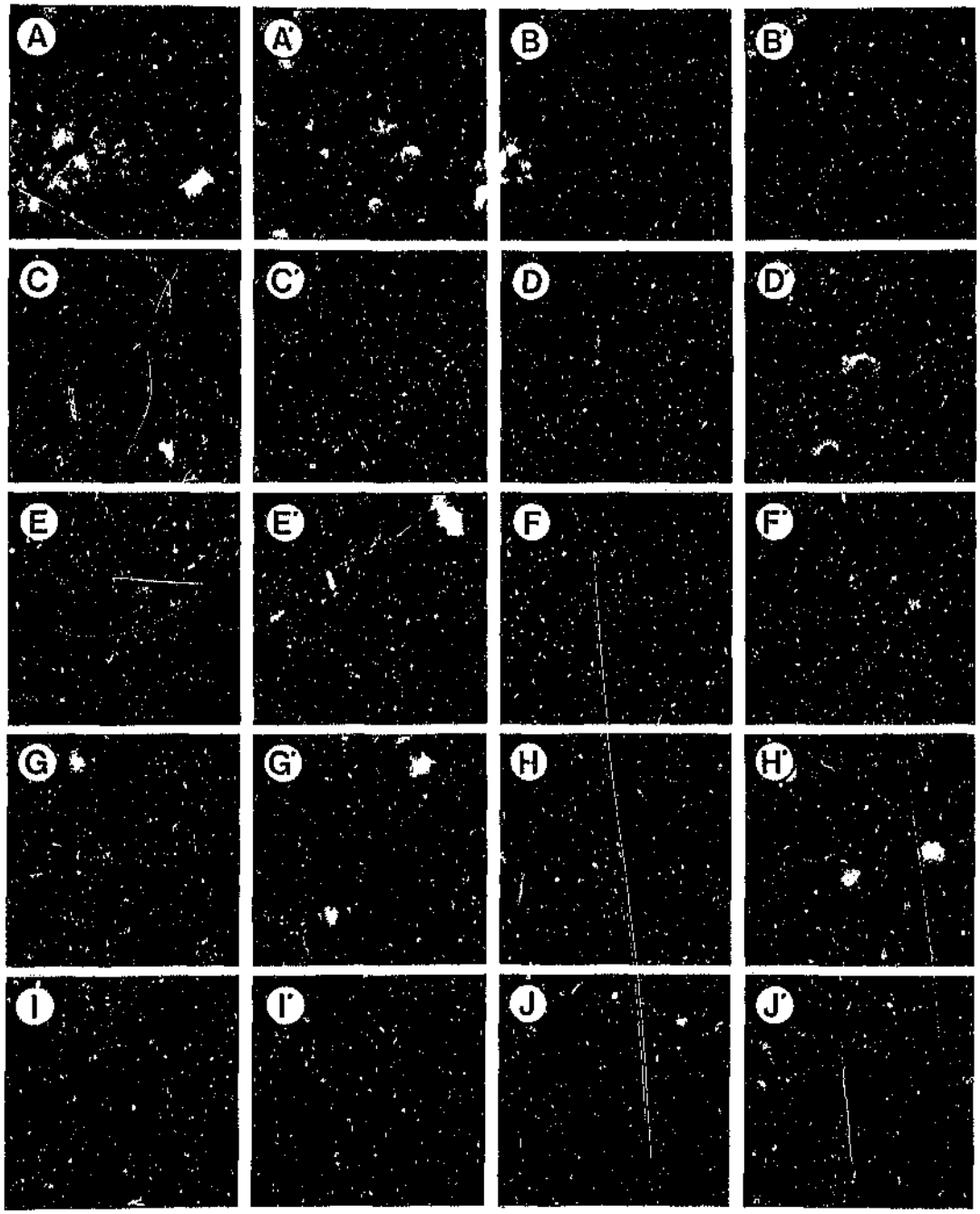

Figure 3.12. See figure legend on the following page.

Figure 3.12. Subcellular localisation of NPM-ALK, TPR-MET, and engineered mutant proteins. Cells were immunostained using either the ALK-specific monoclonal antibody ALK-1 (neat) (all panels except D, D', F and F') or the h-MET (C28) antibody (1:500). Simultaneous staining with DAPI was performed to visualize the nuclei of the cells (panels A' to J'). (A, A') t(2;5)-positive lymphoma cell line SUP-M2, (B, B') t(2;5)-negative chronic myelogenous leukemia cell line K562, (C, C') NPM-ALK, (D, D') TPR-MET, (E, E') TPR-ALK, (F, F') NPM-MET, (G, G') Δ^{MB}NPM-ALK, (H, H') Δ^{64}NPM-ALK, (I, I') $\Delta^{95\text{-}103}$NPM-ALK, (J, J') $^{M5/7/0C}$NPM-ALK. All cDNA constructs were expressed in COS-7 cells (panels C, C' to J, J').

localisation, the subcellular location of the NPM-MET protein was determined. Because TPR-MET is completely cytoplasmic (figure 3.12 D), localisation of NPM-MET to the nucleus would have to be dependent upon the NPM portion of this fusion. Immunofluorescent staining of COS-7 cells expressing NPM-MET revealed a pattern of both cytoplasmic and nuclear staining (figure 3.12 F) qualitatively identical to that observed with wild-type NPM-ALK (figure 3.12 C). Lastly, despite its ability to transform fibroblasts as efficiently as NPM-ALK, the TPR-ALK mutant protein was localised within the cytoplasm (figure 3.12 E).

4. DISCUSSION

The fusion of a nucleolar protein, NPM, to a tyrosine kinase, ALK, raises novel questions about how such a chimaeric protein might function (Sawyers and Denny, 1994). Because the normal ALK receptor is expressed only in neural tissues, clearly one result of this gene fusion is the ectopic expression of *ALK* in lymphoid cells, driven by the strong *NPM* promotor (Morris et al., 1996). It was demonstrated that the NPM-ALK protein possesses constitutive phosphotransferase activity in the absence of any mutations within the ALK segment and this indicates that the NPM moiety serves as an activating sequence. Lastly, although the nuclear localisation signals of NPM are not incorporated into the chimaera, the prior demonstration that the normal NPM protein exists as a homo-oligomer shuttling proteins between the nucleus and the cytoplasm suggests that NPM-mediated nuclear localisation of NPM-ALK could expose the fusion protein to a new range of kinase substrates required for cellular transformation (Adachi et al., 1993; Borer et al., 1989; Chan et al., 1989; Dumbar et al., 1989; Fankhauser et al., 1991; Schmidt-Zachmann and Franke, 1988; Schmidt-Zachmann et al., 1987). The presence of NPM-ALK within the nucleus could potentially also alter normal cell growth by interfering with other functions of normal NPM, which are reported to include

roles in the enhancement of chain elongation during DNA replication, ribonuclease activity, and regulation of the activity of certain transcription factors such as the YY1 protein and an incompletely characterised factor that controls the expression of the p120 proliferation-associated nucleolar protein (Chatterjee et al., 1991; Feuerstein et al., 1990; Herrera et al., 1995; Inouye and Seto, 1994; Peter et al., 1990). These potential functions for the NPM portion of NPM-ALK have been addressed in this thesis by assessing the effect on the transformation potential of the chimaera of various NPM deletions or substitutions.

Previous analyses of truncated oncogenic protein tyrosine kinases have identified two apparent types of fusion partners. Fusion partners like the non-muscle tropomyosin (*TPM3*) or translocated promotor region (*TPR*) genes found initially in the *TPM3-TRK* and *TPR-MET* oncogenes, respectively, appear to mediate dimerisation of the fusion proteins, resulting in constitutive tyrosine kinase activity of their protein tyrosine kinase moieties (Martin-Zanca et al., 1986; Park et al., 1986; Ullrich and Schlessinger, 1990). Although essential for transforming activity, these segments can be interchanged with other motifs that result in protein-protein interaction without loss of transforming activity of the resulting chimaeras. For example, recombination of *TRK* kinase domain sequences during NIH3T3

transfection experiments with at least 14 different unrelated 5' end DNA sequences produces transforming chimaeras (Kozma et al., 1988; Oskam et al., 1988). Oncogenic fusion protein tyrosine kinases in naturally occurring human tumours can likewise be comprised of unrelated amino-terminal residues fused to a common kinase partner, an example being the *RET* papillary thyroid carcinoma oncogenes that can be formed by fusion with dimerisation motifs encoded by either the *D10S170* or *RIα* genes (Bongarzone et al., 1993; Grieco et al., 1994; Minoletti et al., 1994). Furthermore, these various activating sequences are not necessarily specific to a particular kinase partner but may generate the biologic function of multiple effector genes. For example, in addition to TPR-MET, TPR sequences are fused to the TRK kinase domain in human papillary thyroid tumours and form the amino terminus of an oncogenic RAF serine/threonine kinase in experimentally-induced rat tumours (Greco et al., 1992; King et al., 1988). Aside from dimerisation, there is no evidence that these sequences serve additional functions important for oncogenesis. The amino-terminal fusion of BCR residues to the truncated ABL protein tyrosine kinase stands in contrast to the fusions that occur with partners like TPM3 or TPR. In addition to mediating homo-oligomerisation of BCR-ABL and hetero-oligomerisation with normal BCR, the BCR residues in these chimaeras perform multiple functions required for BCR-ABL biologic activity, including

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(a) interaction with the SH2 domain of the substrate GRB-2 to activate the RAS pathway, (b) intrinsic serine/threonine kinase activity, (c) binding to the ABL SH2 domain in a phosphoserine/phosphothreonine-dependent manner, and (d) relocalisation of ABL from its normal predominantly nuclear location to the cytoplasm (Campbell et al., 1990; Maru and Witte, 1991; McWhirter et al., 1993; Pendergast et al., 1989; Pendergast et al., 1991; Pendergast et al., 1993). In addition, differing portions of BCR present in $p185^{BCR-ABL}$ in acute lymphoid leukaemia as compared to $p210^{BCR-ABL}$ in chronic myelogenous leukaemia result in five-fold higher kinase activity and greater transforming ability of the p185 variant (Lugo et al., 1990). Substitution of BCR with unrelated sequences of comparable length renders the resulting fusion non-transforming, as does mutation of the sequences known to mediate the above-mentioned functions of BCR (Muller et al., 1991).

The structure-function studies that are detailed here indicate that NPM sequences of NPM-ALK contain an oligomerisation domain, the function of which is absolutely required for the *in vivo* kinase activity of the chimaera. The nature of the NPM oligomerisation motif remains to be determined; for example, computer-assisted analysis (COILS 2.0 (Lupas et al., 1991)) of the NPM amino acid sequence failed to identify sequence similarity to other oncogenic fusion partner proteins such as TPR, TPM3, BCR or TFG that

contain coiled-coil domains that mediate oligomerisation (Greco et al., 1995; McWhirter et al., 1993; Rodrigues and Park, 1993). The demonstration that any of three non-overlapping deletions that together encompass the NPM portion of the chimaera inhibit complex formation indicates that this entire segment is necessary for oligomerisation to occur. The portion of NPM present in NPM-ALK shares 47% amino acid identity with the amino-terminus of nucleoplasmin, a pentameric protein that binds histones and transfers them to DNA during nucleosome assembly (Dingwall et al., 1987; Dingwall et al., 1982). This segment of nucleoplasmin is contained within an amino-terminal core peptide released after proteolytic digestion that retains its ability to form pentamers (Dingwall and Laskey, 1990). These structure-function studies suggest that the 118 amino acid segment of NPM, and the corresponding segment of nucleoplasmin, represent the minimal motif required for the homo-oligomerisation of these proteins. Thus, like other kinase fusion partners that have been characterised previously, NPM is ubiquitously expressed and contains an oligomerisation domain (Rodrigues and Park, 1994). Although it was not unequivocally excluded that other potential functions of the NPM segment of NPM-ALK could be important for transformation, the fact that a completely unrelated segment from the TPR protein that is known to promote oligomerisation can efficiently activate the

transforming ability of the truncated ALK protein argues that other NPM functions are unlikely.

The subcellular localisation of NPM-ALK suggests that the protein might generate growth regulatory signals not only in the cytoplasm but also within the nuclear/nucleolar compartment. Recent experimental data support a role for nuclear protein tyrosine kinases and their substrates in RNA processing and trafficking, transcriptional control of gene expression and possibly other nuclear processes (Baskaran et al., 1993; Fumagalli et al., 1994; Kipreos and Wang, 1992; Taylor and Shalloway, 1994). In addition, several "cytoplasmic" protein tyrosine kinases including SRC, FER, and FGR are now known to localise in part to the nucleus in some cells or under certain conditions (Wang, 1994). The subcellular location of the engineered NPM-MET protein to the nuclear compartment indicates that the NPM moiety of NPM-ALK can mediate nuclear/nucleolar trafficking (most probably by allowing hetero-oligomerisation with normal NPM), an observation corroborated by the presence within the cytoplasm only of the three NPM deletion mutants Δ^{64}NPM-ALK, $\Delta^{65\text{-}103}$NPM-ALK and Δ^{MB}NPM-ALK. Whether the lack of nuclear localisation, independent of their failure to homo-oligomerise and acquire constitutive kinase activity, is responsible in part for the inability of these three mutants to transform cells cannot be

determined. However, the efficient transformation of Fr3T3 fibroblasts by the cytoplasmic TPR-ALK protein indicates that nuclear localisation of an activated ALK protein is not necessary for the malignant conversion of fibroblasts. Furthermore, the recent identification in anaplastic large cell lymphoma of a rare variant t(1;2)(q25;p23) translocation that joins activating sequences other than NPM to the ALK protein, producing a fusion kinase restricted to the cytoplasm, indicates that nuclear localisation is also not required for lymphoid cell transformation (Pulford et al., 1996).

The signalling substrates utilised by NPM-ALK in transformation are not yet known. The presence in the chimaeric protein of a potential phosphotyrosine-binding (PTB) motif ($NPTY^{567}$) prompted the examination of the interaction of the SHC protein tyrosine kinase substrate with wild-type NPM-ALK and with an engineered mutant, Δ^{C154}NPM-ALK, lacking its carboxy-terminal 154 residues (including the NPTY motif) (Pelicl et al., 1992; Sun et al., 1991; van der Geer and Pawson, 1995). Whereas wild-type NPM-ALK physically associated with and phosphorylated SHC, Δ^{C154}NPM-ALK was unable to do so, suggesting that SHC binds the $NPTY^{567}$ motif of the chimaera. Despite its inability to interact with SHC, the Δ^{C154}NPM-ALK protein efficiently transformed fibroblasts, indicating that SHC is not an essential substrate for transformation and suggesting that another substrate with

overlapping function such as GRB-2 may substitute for SHC activity (Lowenstein et al., 1992). In a report published during the preparation of this thesis, Fujimoto and co-workers independently described the cloning of the *NPM-ALK* cDNA and addressed the role of SHC and IRS-1 in NPM-ALK-mediated transformation using mutant constructs in which either or both tyrosine residues 156 and 567 were changed to phenylalanine (Fujimoto et al., 1996). In agreement with our data, SHC was found to bind to the $NPTY^{567}$ motif. Interestingly, both of the single point mutation chimaeras, as well as the double mutant, transformed cells efficiently, indicating that neither IRS-1 nor SHC are essential for NPM-ALK-induced transformation. In addition, wild-type NPM-ALK and all three point mutant chimaeras were shown to efficiently associate with GRB-2.

Recent studies indicate that the role of NPM in malignancy is not restricted to its involvement in the t(2;5). Analysis of the rare t(5;17) found in acute promyelocytic leukaemia has recently shown that this rearrangement results in the fusion of the 5' end of *NPM* to the retinoic acid receptor alpha (*RARα*) gene to produce a chimaeric gene (Redner et al., 1996). The t(5;17) generates two chimaeric NPM-RARα proteins - NPM_s-RARα, which contains the same NPM residues that are present in NPM-ALK, and NPM_L-RARα, which possesses an additional 50bp from the *NPM* coding sequence plus 79bp of

coding sequence of unknown derivation. Both of these fusions contain the same portion of RARα that is present in the more common promyelocytic leukaemia (PML)-RARα and promyelocytic leukaemia zinc finger (PLZF)-RARα chimaeric proteins (Zelent, 1994). The functions of NPM in NPM-RARα remain to be determined, but it is possible that the motif mediates homo-dimerisation (as does the PML segment of PML-RARα) resulting in altered transcriptional properties of RARα (Doucas et al., 1993; Kastner et al., 1992; Pandolfi et al., 1991; Zelent, 1994). An alternative, but not mutually exclusive, possibility is that NPM and NPM-RARα heterodimers could localise to specific regions within the nucleus, altering the subcellular location of RARα binding partners and thus indirectly affecting normal RARα function (Weis et al., 1994). It was determined that the t(3;5)(q25.1;q34) rearrangement that is found in approximately 0.5-1% of all myelodysplastic syndromes and acute myeloid leukaemias also involves NPM, producing an in-frame fusion of 5' end *NPM* sequences to those of a novel gene, that was named myelodysplasia/myeloid leukaemia factor (*MLF1*), whose deduced amino acid sequence contains no regions of homology with previously characterised proteins (Yoneda-Kato et al., 1996). NPM-MLF1 contains 58 more NPM amino acids than do NPM-ALK and NPM_s-RARα fusion proteins, including one of the two NPM nuclear localisation signals and most of the glutamic and aspartic acid residues present in the two acid amino acid

clusters of NPM. Immunostaining experiments indicate that MLF1 is normally located in the cytoplasm, whereas NPM-MLF1 is targeted to the nucleus, with highest expression in the nucleoli. Like NPM-ALK, NPM-MLF1 can heterodimerise with NPM (S.W. Morris unpublished), probably contributing to this relocalisation of the MLF1 protein. Structure-function analysis of NPM-RARα and NPM-MLF1 similar to those performed here for NPM-ALK should be of significant interest; in light of the fusion of NPM to three functionally diverse proteins, it is likely that the functions of the NPM segment in each of these three chimaeras will be found to be partially overlapping.

5. CONCLUSIONS

Experiments were designed to investigate the transforming ability of NPM-ALK, and the role of NPM fusion sequences in the oncogenic activation of NPM-ALK.

The oncogenic potential of NPM-ALK was demonstrated in several ways. Rodent fibroblasts stably expressing the chimaeric protein were able to form anchorage-independent colonies in soft agar. In addition, fibroblasts infected with retrovirus prepared utilising the *NPM-ALK* retroviral construct were able to form densely packed, morphologically transformed cells that coalesced upon further growth to form sheets of abnormal cells. Furthermore, mice injected with clonal rodent fibroblasts stably expressing NPM-ALK developed tumours at the site of injection as soon as five days post-injection. Future studies could perhaps investigate the oncogenic potential of this fusion protein in haematopoietic cells. The availability of murine haematopoietic cell lines that grow only in the presence of interleukin-3 makes this possible. The cells expressing NPM-ALK would be rendered interleukin-3-independent in the case of transformation due to the chimaera.

It was demonstrated that the intrinsic kinase activity of ALK is required for NPM-ALK-mediated transformation by the construction of a kinase-defective mutant in which the invariant lysine residue in the ATP-binding region of the catalytic domain of protein kinases was changed to an arginine. As expected, this mutant failed to transform fibroblasts. In addition, it was shown that transformation by NPM-ALK does not require the SHC binding site located in the carboxy terminus of the ALK portion in NPM-ALK. Further investigation is required to determine which substrates may be required for the transformation by NPM-ALK. This could be achieved by mutating potential "substrate" sites and subsequently testing the transforming ability of these mutant constructs.

An intact NPM segment is absolutely required for NPM-ALK-mediated oncogenesis, as indicated by the observation that three different NPM-ALK mutant proteins lacking non-overlapping portions of the NPM segment each failed to form oligomeric complexes, lacked kinase activity *in vivo*, and failed to transform cells. In contrast, NPM-ALK was shown to homo-oligomerise *in vivo* and heterodimerise with normal NPM-ALK in addition to exhibiting kinase activity *in vivo*. Furthermore, it was found that the portion of NPM present in the fusion protein, shares 47% amino acid identity with the amino-terminus of nucleoplasmin. These structure-function studies suggest that the

118 amino acid segment of NPM, and the corresponding segment of nucleoplasmin, represent the minimal motif required for the homo-oligomerisation of these proteins. This aspect requires further experimentation to determine more precisely the nature of this minimal motif.

Immunostaining experiments of a t(2;5)-containing lymphoma cell line revealed NPM-ALK to be localised within both the cytoplasmic and nuclear/nucleolar compartments. The normal shuttling function of NPM, together with the observations that NPM and NPM-ALK heterodimerise, suggest that the nuclear location of the chimaera results from NPM-mediated protein translocation since the nuclear localisation signals of NPM are not present in NPM-ALK. It was found that none of three different NPM-ALK proteins lacking non-overlapping portions of the NPM segment was present in the cell nucleus. However, NPM could be replaced in the fusion protein with the portion of the TPR protein that activates the TPR-MET fusion kinase by mediating dimerisation through its leucine zipper motif. This engineered TPR-ALK hybrid protein, which transformed cells with essentially equivalent potency to NPM-ALK, was localised within the cytoplasm of cells. These data indicate that the nuclear/nucleolar localisation of NPM-ALK, which probably occurs because of transport via shuttling activity of NPM, is

not required for oncogenesis. Furthermore, the activation of the truncated ALK protein by a completely heterologous oligomerisation domain suggests that the functionally important role of the NPM segment of NPM-ALK in transformation is restricted to the formation of kinase-active oligomers and does not involve the alteration of normal NPM function. Since the presence of NPM-ALK in the nucleus/nucleoli does not appear to be involved in transformation, the function of the chimaeric protein in these cellular compartments, if any, has yet to be determined.

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