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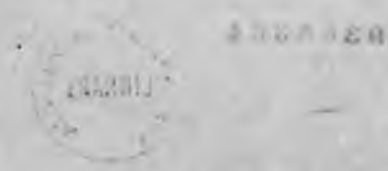
CHARACTERISATION AND EXPRESSION STUDIES OF A XENOPUS LAEVIS
REL HOMOLOGUE.

A thesis submitted for the degree of Doctor of Philosophy.

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December, 1991.



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Dedication.

To Mum and Dad

Declaration.

All the results presented in this thesis were obtained by the author, apart from those indicated in the text, or specified below.

All oocyte/embryo injections and dissections were performed by Hugh Woodland. Phenotypic and histological analysis of embryos was in collaboration with Hugh Woodland. Immunochemical analysis of oocyte and embryo sections was in collaboration with Elizabeth Jones.

All sources of information have been acknowledged by means of reference.

None of the work contained in this thesis has been used for any previous application for a degree.

Abbreviations.

aa	amino acid
Ac	acetate.
ATP, dATP,	adenosine triphosphate, deoxyadenosine
ddATP	triphosphate, dideoxyadenosine triphosphate.
bp	base pair.
BCIP	4-chloro-3-indoyl phosphate.
cDNA	complementary DNA.
Ci	Curie.
CIAP	calf intestinal alkaline phosphatase.
CTP, dCTP,	cytidine triphosphate, deoxycytidine
DAG	diacyl glycerol.
ddCTP	triphosphate, dideoxycytidine triphosphate.
ddH ₂ O	didistilled water.
DMF	dimethyl formamide.
DNA	deoxyribonucleic acid.
DNase	deoxyribonuclease.
DOC	deoxycholate.
DTT	dithiothreitol.
dTTP,	Thymidine triphosphate, deoxythymidine
ddTTP	triphosphate.
EDTA	Ethylene diamine tetra acetic acid.
GEMSA	gel electrophoretic mobility shift assays.
GSH	glutathione.

GTP, dGTP,	guanosine triphosphate, deoxyguanosine
ddGTP	triphosphate, dideoxyguanosine triphosphate.
HACoCl ₃	hexamino cobalt trichloride.
HEPES	N-[2-Hydroxyethyl] piperazine -N'-[2-ethane sulphonic acid].
IPTG	isopropyl-B-D-thiogalactosidase.
Kb	kilobase pairs.
KD	kilodaltons.
LPS	lipopolysaccharide.
l	litre.
MES	2 [N-morpholino] ethane sulphonic acid.
ml	millilitre.
MOPS	3-(N-morpholino) propanesulfonic acid.
MS222	3-aminobenzoic acid ethyl ester.
NAC	N-acetyl-cysteine.
NBT	nitro-blue tetrazolium.
ng	nanogram.
NLS	nuclear localisation signal.
ORF	open reading frame.
pfu	plaque forming units.
pg	picogram.
PKC	protein kinase C.
PMA	12-phorbol-13-myristate acetate.
Pol	polymerase.

RH	rel homologous.
RNA	ribonucleic acid.
RNase	ribonuclease.
ROI	reactive oxygen intermediates.
rpm	revolutions per minute.
rRNA	ribosomal RNA.
S	Svedberg.
SDS	sodium dodecyl sulfate.
SEP	sperm entry point.
TCA	trichloroacetic acid.
TE	Tris (10mM pH8)-EDTA (1mM).
TFB	transformation buffer.
TNF	tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane.
tRNA	transfer RNA.
ug	microgram.
ul	microlitre.
UTP	uridine triphosphate.
v/v	volume/volume.
w/v	weight/volume.
wt	wild-type
X-gal	5-Bromo-4-chloro-3-indoyl-B-D-galactopyranoside.

Introduction

CHAPTER 1 : INTRODUCTION

1.1 Regional organisation during early development of Xenopus laevis.

Homologues of developmentally important regulatory genes from other species have been found to be expressed in early amphibian development. The Drosophila dorso-ventral polarity gene, dorsal, is involved in the formation of ventral structures and dorsal⁻ mutants arise from a default state where only dorsal structures are formed. A Xenopus homologue of dorsal may play a crucial role in interpreting some of the earliest embryonic signals. This section, therefore, outlines the events of early amphibian development up to the point of neurulation and discusses in detail some of the proposed molecules involved in these early signals.

1.1.1 Regionalisation within the egg.

Early amphibian development consists of stage-specific morphogenetic events by which an oocyte is converted into an egg and then embryo. The first kinds of cell diversity that arise from the fertilised egg are thought to result from cytoplasmic factors that are unevenly localised in eggs, and are therefore differentially distributed to blastomeres in cleavage embryos (Gerhart et al., 1984).

The Xenopus oocyte is a large cell (1.3mm in diameter) in which components are unevenly distributed, even to the

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naked eye. The brownish animal hemisphere contains a large nucleus and a layer of melanin granules near the plasma membrane. The yellowish vegetal hemisphere consists of a mass of membrane-bound yolk platelets and germ plasm granules at its pole. The animal-vegetal axis of rotational symmetry connects the poles of these hemispheres and the oocyte is radially symmetrical about this axis. The surface of the oocyte is characterised by a cortical layer of microvillus-associated microfilaments and of cyokeratin intermediate filaments. The transition of the oocyte to unfertilised egg preserves the organisation of the oocyte and prepares the cortex for fertilisation (Gerhart et al., 1984).

Fate mapping and isolation experiments have led to the conclusion that the organisation of the egg is predictably related to the tissue organisation of the embryo, though not in an entirely deterministic way (eg. Dale and Slack, 1987a; Moody, 1987; Nieuwkoop, 1969). The animal and vegetal halves of the egg constitute primary cytoplasmic localisations of the egg principally forming the ectodermal (future nervous system and skin) and endodermal (future digestive and respiratory) systems respectively. However, without cell interactions between these two fundamental constituents of the embryo, only epidermis and endoderm can form as default states; most importantly mesoderm is absent. (reviewed by Gurdon, 1989; Smith, 1989; Woodland, 1989).

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1.1.2 Dorso-ventral polarisation.

A cytoplasmic rearrangement during the first cell cycle following fertilisation, triggered and orientated by sperm entry, specifies the dorsoventral axis of the amphibian embryo. Evidence that dorsoventral (DV) polarity is caused by this 30° rotation of the entire thin outer cortex relative to the deeper cytoplasm, includes experiments where vegetal halves of eggs are irradiated with ultraviolet light and the rotation is blocked. The resulting embryos are radial and lack all dorsoanterior structures (Vincent and Gerhart, 1987). The direction of this subcortical rotation eventually defines the dorsal midline of the embryonic axis. The egg surface contracts toward the fertilising sperm causing a relative displacement of the surface to the internal cytoplasm and the blastopore lip forms opposite the sperm entry point (SEP). These internal cytoplasmic rearrangements normally take place in response to sperm entry: however, the prospective DV axis can be experimentally altered by gravity after fertilisation - this is carried out by rotating the egg in a solution of Ficoll (a polysaccharide which can remove the water from the space below the vitelline membrane, so preventing the egg rotating within the membrane) so that the animal-vegetal axis is not vertical. Internal cytoplasmic rearrangements of the immobilised tipped egg then take place in response to the gravitational field rather than the sperm. Elinson and

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Rowning (1988) used immunocytochemical and electron microscopy examination of eggs undergoing rotation to reveal an extensive array of parallel microtubules covering the vegetal hemisphere of the egg. These microtubules were 1-3um from the plasma membrane and were aligned parallel to the direction of rotation. The presence of these microtubules, the sensitivity of rotation to ultraviolet irradiation and colchicine treatment (which also inhibits the formation of the microtubules) and rotation itself all coincide temporally (between 0.5-0.8 where 0 is the time of insemination and 1.0 is the time of first cleavage). The conclusion from these experiments is that the polymerising parallel microtubules served as tracks for cytoplasmic rotation by aligning in the direction of the initial shear (caused by the sperm aster) and by enhancing shearing in the same direction thereby reinforcing the initial movement and thus specifying the dorsoventral axis of the embryo (Elinson and Rowning, 1988).

1.1.3 Regional organisation during cleavage and blastula stages.

During the morula and blastula stages of development one of the first embryonic inductive cellular interactions takes place, referred to as mesoderm induction. Embryonic induction describes an interaction between one (inducing) tissue and another (responding) tissue as a result of which the responding tissue undergoes a change in its direction

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of differentiation (Gurdon, 1987). In the case of mesoderm induction, the vegetal cells (presumptive endoderm) induce the overlying animal cells (presumptive ectoderm) to form mesodermal cells at the junction between the animal and vegetal hemispheres of the embryo, the marginal zone. Explanted cells from the animal hemisphere (animal caps) form epidermal cells when cultured in isolation, but these same cells will form mesodermal tissue when placed in contact with vegetal (endodermal) cells (Nieuwkoop, 1969). These explantation experiments suggest that the signal produced from the vegetal cells is a secreted signal. Furthermore, neither blocking of gap junctions by anti-gap junction antibody (Warner and Gurdon, 1987), nor separation of the explants by a nucleopore filter of sufficient size to exclude cytoplasmic processes (Grunz and Tacke, 1986) are able to inhibit mesoderm induction, indicating the involvement of an intercellular signal that is not attached to the cell surface or cytoplasm.

Nieuwkoop (1969) further demonstrated, with the axolotl, that the type of mesoderm that forms in animal-vegetal combinations depends on the origin of the vegetal inducing cells. Dorsovegetal cells tended to induce dorsal cell types such as notochord and muscle while lateral and ventral vegetal blastomeres induced blood, mesenchyme and mesothelium. The pattern of cell types in the mesoderm is therefore wholly determined by information derived from underlying vegetal blastomeres.

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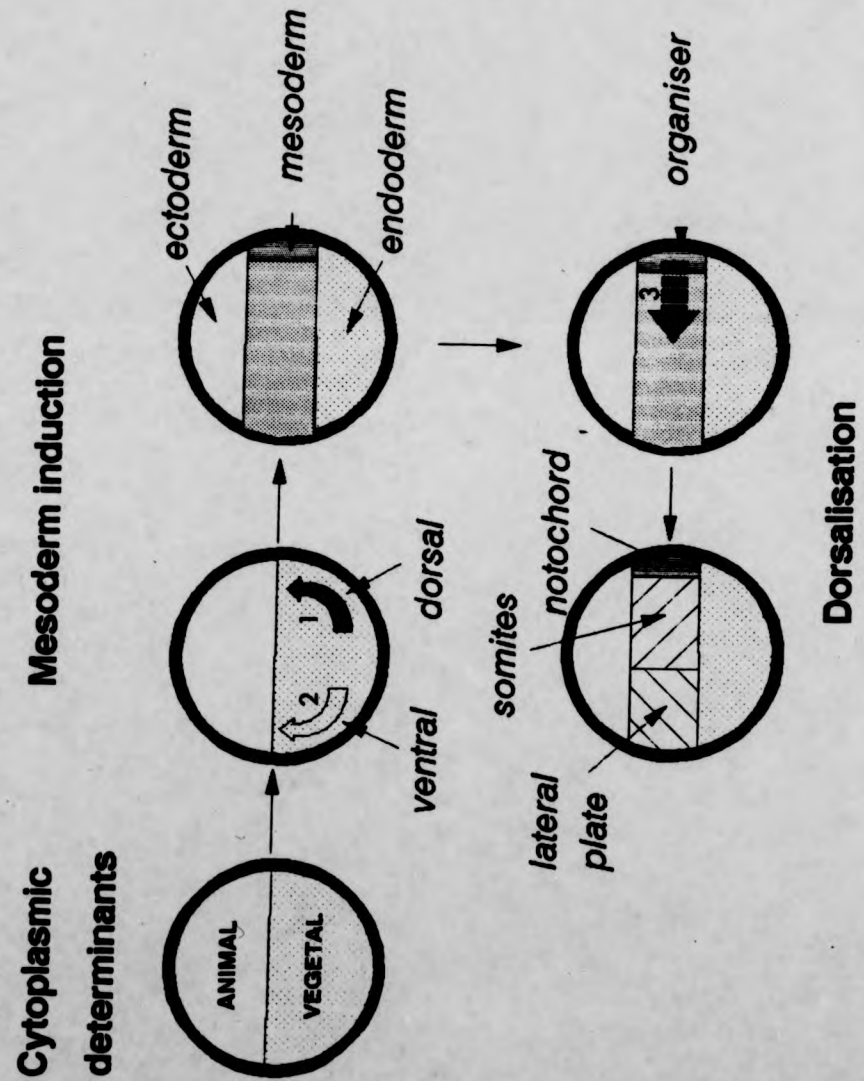
1.1.4 The 3-signal model of mesoderm induction and the proposed molecules representing these signals.

Nieuwkoops' work on the axolotl was repeated on Xenopus by Dale and others (1985) who concluded that the signals emitted by dorsal and ventral vegetal cells are not finely graded. A model was proposed in which a signal on the dorsal side induces notochord and a small amount of muscle; a signal on the ventral side induces blood, mesenchyme and mesothelium. However, a third signal, which emanates from the newly induced dorsal mesoderm, acts within the prospective mesodermal germ layer and is responsible for further subdivisions of the mesoderm (see figure 1). This signal 'dorsalises' adjacent ventral mesoderm and directs it away from forming blood to become muscle instead. Experiments performed by Slack and Forman (1980) provided evidence that the intermediate states of the marginal zone are generated by this third 'dorsalising' signal. In isolation dorsal marginal zone tissue produced notochord, some muscle and neural tissue, while ventral marginal zone cells produced blood, mesenchyme and mesothelium; however, in combinations of the two regions the ventral marginal zone formed large amounts of muscle, that is, it was 'dorsalised'. In combination, the ventral marginal zone failed to form either blood or mesothelium, thus the 'dorsalisation' was incomplete and involved promotion of ventral tissue to an intermediate status. There was no reciprocal effect of the ventral on the dorsal zone; that

Figure 1.

The 3-signal model of mesoderm induction.

The animal and vegetal halves of the egg constitute primary and cytoplasmic localisations of the egg. If there were no cell interactions between these constituents of the embryo, only epidermis (from ectoderm produced from the animal half) and endoderm (from the vegetal half) would form as default states. Signal 1 represents the dorsovegetal signal, which induces notochord and a small amount of muscle. Signal 2 represents the ventrovegetal signal, which induces blood, mesenchyme and mesothelium. Signal 3, which emanates from within the newly induced dorsal mesoderm, acts within the newly produced mesoderm and gives rise to further subdivisions of the mesoderm. (Adapted from Woodland, 1989).



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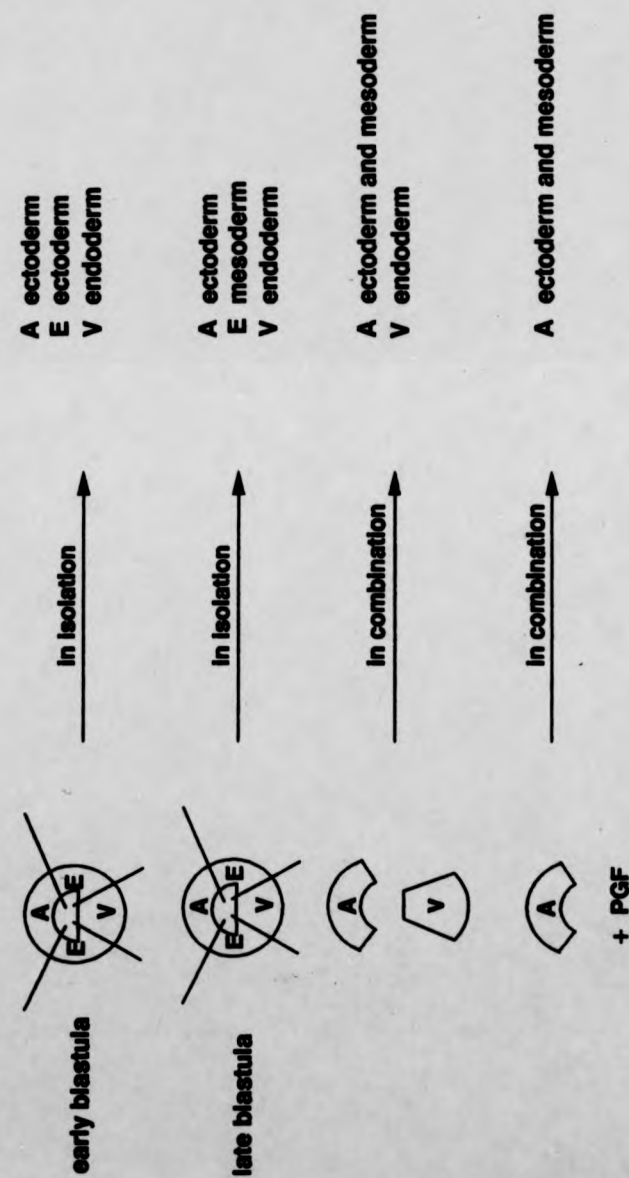
is, while ventral tissue may be dorsalised, dorsal tissue may not be 'ventralised'. The initial dorsoventral organisation of mesoderm-inducing stimuli is therefore crude, so further interactions are required to produce the final detail. These interactions include a 'dorsalising' activity which is confined to a restricted part of the dorsal marginal zone - an area which is wider than the prospective notochord but is apparently graded from a high point at the dorsal midline (Dale and Slack, 1987b).

For many years, the effect of the vegetal pole cells in mesoderm induction has been mimicked by many heterologous factors such as carp swim bladder, guinea pig bone marrow and heated HeLa cells; these factors are clearly not from early embryonic stages nor from Xenopus. However, since different classes of molecules can mimic each of the signals in the three-signal model of mesoderm induction (figure 1), it does suggest that three independent classes of response are involved. A more plausible molecular representative of mesoderm inducing factors which are actually involved in Xenopus morphogenesis stemmed from the discovery that members of growth factor families are able to induce mesoderm (Smith, 1989)(see figure 2). Peptide growth factors were initially characterised using either cultured mammalian cell systems (PDGF, TGF) or in vivo assays (EGF) as small, soluble polypeptides capable of regulating cellular physiology (reviewed by Whitman and Melton, 1989a). These factors can be grouped into a fairly

Figure 2.

Schematic representation of mesodermal induction in isolation/recombination experiments.

Animal, vegetal or equatorial tissue is dissected from blastulae and allowed to differentiate in culture. Mesoderm is formed from: the equatorial region of late blastulae; animal cap tissue recombined with vegetal tissue; animal cap tissue cultured in the presence of mesoderm inducing factors, eg. peptide growth factors (PGF) such as Activin and FGF.
(Adapted from Whitman and Melton, 1989).



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small number of families, members of which are closely related structurally and may interact with similar or identical receptors. Peptide growth factors that have been implicated in the induction of mesoderm in *Xenopus*, referred to as mesoderm inducing factors (MIFs), belong to the fibroblast growth factor (FGF) family (table 1) and the transforming growth factor beta (TGF- β) family (table 2)

The usual assay for testing mesoderm inducing activity is to culture isolated animal pole tissue in dilute solutions of the factors under investigation. In the absence of MIFs only epidermis is formed but in their presence mesodermal cell types arise. Both groups of MIFs are active at micromolar concentrations but they give rise to different types of tissue. TGF- β -related MIFs induce mesodermal cell types such as notochord, muscle, kidney, mesenchyme and mesothelium (Smith *et al.*, 1988), whereas FGF can induce all of these cell types except the most dorsal mesoderm, notochord (Cooke, 1989). Thus, it has been suggested that signal 1 in the model of mesoderm induction is TGF- β like and signal 2 is FGF-like (figure 1). The original motivation for this model came from the fact that TGF- β 1 did nothing on its own, but potentiated the effect of FGF. However, since some members of the TGF- β family can induce the whole range of tissues, a minimal hypothesis requires only one agent.

Significantly, some of these peptide growth factors or their receptors are structurally or functionally related to

Table 1.

Properties of the FGF-related family of Growth Factors.
 Adapted from Cross and Dexter (1991). Cell 64: 271-280.

Growth Factors	Structure	Source	Known Targets	Receptors
FGF-1 and FGF-2 (acidic and basic FGF respectively)	About 16-17 kd. Sometimes larger forms. No consensus signal peptide. 55% identical. Also related to other FGFs and interleukin 1 family.	Low mRNA levels in wide range of normal and transformed cells. Proteins widely distributed, associated with extracellular matrix.	Variety of endothelial, epithelial, mesenchyme and neuronal cell types.	FGF-1 (105 kd) and FGF-2 (130 kd) receptors both tyrosine kinases. High cross-reactivity of FGF-1 and FGF-2 binding
FGF-3 (<u>int-2</u>)	27-32 kd alternative translation products of <u>int-2</u> proto-oncogene.	mRNA in mouse embryonic tissues, brain, testes, mouse mammary tumour, and teratocarcinoma cells.	Unknown.	Unknown.
FGF-4	19 kd glycoprotein of <u>hst</u> (human) and or <u>ks3</u> (mouse) proto-oncogenes.	Unknown.	Vascular endothelial cells, fibroblasts.	Unknown.
FGF-5	26 kd glycoprotein.	Unknown.	Fibroblasts.	Unknown.

Table 2.

Properties of some members of the TGF- β family.

1. Massague (1987). Cell 49: 437-438.
2. See 1.
3. See 1.
4. Padgett et al., (1987). Nature 325: 81-84.
5. Lyons et al., (1989). Proc. Natl. Acad. Sci. USA 86: 4554-4558.
6. Whitman and Melton (1987). Annu. Rev. Cell. Biol. 5: 93-117.
7. See 6.

* Activin B has not been isolated from natural sources.

Group	Types of subunits	Bioactive dimers composition (produced from inactive precursor)	Name	Source	Distribution and function
1. TGF- β	$\beta 1$ $\beta 2$	($\beta 1$) ₂ ($\beta 2$) ₂ ($\beta 1, \beta 2$)	TGF- $\beta 1$ TGF- $\beta 2$ TGF- $\beta 1.2$	Mammals	Widespread tissue distribution. Blocks further proliferation of differentiated cells, such as; epithelial, lymphoid and endothelial cells.
2. Inhibins and Activins	α βA βB	($\alpha, \beta A$) ($\alpha, \beta B$) (βA) ₂	Inhibin A Inhibin B Activin A (=PIF-EDF) (=XTC-MIF) Activin AB	Mammals Mammals Mammals Xenopus Mammals of erythroleukemia cells (but	Inhibins inhibit FSH secretion from the anterior pituitary and suppress differentiation of erythrocyte leukemia cells. Activins stimulate FSH secretion and induce differentiation of erythroleukemia cells (but activin AB inhibits differentiation of these cells. Activin B is thought to have a role in the induction of axial pattern in vertebrates.
3. Müllerian Inhibitor	MIS	Probably cleaved from (MIS) ₂ precursor.	MIS	Mammals	
4. Deca-pentaplegic	DPP-C	Probably occurs as a dimer after processing and secretion of precursor molecule	DPP-C	<u>Drosophila</u>	Involved in determination of dorsal structures in the developing embryo and in correct morphogenesis of the imaginal discs.

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developmentally important regulatory genes. For example, the homeotic genes Notch, Delta (Drosophila) and lin-12 (nematode), which participate in the differential specification of epidermal and neurogenic tissue share domains of sequence homology to EGF (Greenwald, 1985; Knust et al., 1987). Decapentaplegic (a Drosophila gene complex) which is involved in dorsoventral specification and imaginal disc morphogenesis is related to TGF- β and the vegetally localised Vg-1 mRNA of Xenopus (Padgett et al., 1987).

A TGF- β related factor, XTC-MIF (Smith, 1989), which is a very potent mesoderm-inducing factor secreted by the cell line XTC, has recently been shown to be a Xenopus homologue of mammalian activin A (Smith et al., 1990). The ten terminal amino acids of XTC-MIF are identical to the N-terminal residues in mammalian activin A. Furthermore, presence of mammalian activin A activity has been shown to have a clear and consistent correlation with mesoderm inducing activity of XTC-MIF. Activins were originally characterised as factors causing the release of FSH from anterior pituitary cells (Vale et al., 1986), and subsequently identified as being identical to EDF, an erythroid differentiation factor (Smith et al., 1990), which shares inductive properties and antigenic determinants with PIF (an inducing factor recently isolated from mouse macrophage culture supernatants (P388D1 cell line)). PIF was recently shown to be identical to mammalian

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activin A (Thomsen et al., 1990). Activins, like other active forms of TGF- β -like molecules, (reviewed in Massague, 1987), consist of a dimer of polypeptide chains that have been processed from a larger precursor protein (see table 2). Activins are thought to exist as homodimers of two β_A chains (activin A), heterodimers of a β_A and β_B chain (activin AB) or two β_B chains - although the latter molecule has not been isolated from natural sources. Genes encoding Xenopus activin β_A and β_B chains have been cloned and encode proteins with about 55% similarity. Activin β_B transcripts are first detected in late blastula (Stage 9), whereas activin β_A transcripts are not detected until late gastrula (stage 13) (Thomsen et al., 1990). Porcine activin A (and PIF) have been shown to induce dorsal axial and anterior structures; concentrations of about 15pM can elicit a full inductive response, which is 50-100 fold lower than the minimum concentration of FGF or TGF- β (2 μ M) required for full inductive activity. However, there is evidence that mesoderm induction may antedate MBT, suggesting that Activin B alone cannot be responsible for mesoderm induction.

Cooke (1989) compared the inducing activity of blastocoel roof tissue taken from embryos into which XTC-MIF had been injected, into the blastocoel, with the natural 'Spemann organiser' - a mesodermal component immediately above the dorsal lip - when grafted into the ventral marginal zone. In both cases the grafts resulted in the formation of

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secondary axial patterns. For the XTC-MIF grafts, the completeness and orderliness of secondary patterns depended on graft size and the concentration of XTC-MIF; the optimal conditions were 200ng/ml XTC-MIF and 250 cells of a stage 9 donor. Grafts that were larger than the optimal size resulted in large, but ill-organised, poorly differentiated secondary patterns. For the natural organiser, when grafted in the proper orientation, the larger the graft size, the larger and more vigorous was the new set of cellular movements and therefore the more complete the secondary pattern formed. This contrast in behaviour of the two sources of grafts suggests that further spatial organisation must be an important part of the natural 'dorsal axial' induction. Labelling of graft cells also revealed a difference in character of ectodermal contribution between the experimental and natural organiser tissue. XTC-MIF grafts left a substantial contribution in the non-neural ectoderm overlying the anterior end of the mesodermal pattern they had organised, suggesting that they contain some cells that have not been diverted from their epidermal specification at the time of implantation. Natural organiser grafts contributed only occasionally and minimally to the ectodermal pattern of hosts, which was entirely in ventral spinocaudal parts of second nervous systems; a larger contribution was observed in the lateral plate and laterally derived somite mesoderm of the trunk and tailbud. The dorsal lip grafts may contain cells that

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were already a part of a pattern of bias or induction towards membership of the CNS at the onset of gastrulation of the donor. The difference in these results therefore suggests that the normal dorsal marginal zone achieves more early organisation into subdomains than achieved by homogeneously stimulated experimental tissue, possibly implying that a morphogenetic gradient system is the basis of the organiser effect. The axial patterning of the mesoderm, thus, seems to be determined by the differential competence of cells in the responding tissue as well as by a localised inducer (Sokol and Melton, 1991).

Conditioned medium from COS-1 cells transfected with Xenopus activin β_B has been shown to have a similar level of inducing activity to activin A (XTC-MIF). Furthermore, ectopic expression of activin β_B , as a result of micro-injection of synthetic message, induces a second body axis in host embryos (Thomsen et al., 1990). Activin B, rather than activin A, therefore, is thought to be a key endogenous molecule involved in early induction and axial patterning in Xenopus. The axis of an embryoid formed from an activin-treated animal cap is coincident with the axes of the blastula from which the cap was taken. For example, dorsal but not ventral parts of animal caps give rise to eyes and notochord following induction by activin. Furthermore, injection of activin mRNA into ventral blastomeres can change the ventrally fated mesoderm to a more dorsal fate. Animal caps that have been exposed to a

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homogenous environment of activin, thus, result in the formation of axially patterned embryos, suggesting that an intrinsic polarity of prepattern exists in the animal cap, which is uncovered following induction by activin or XTC-MIF. The patterning of the mesoderm may, therefore, not only be determined by a localised inducer (perhaps a maternal signal) but also by the differential competence of cells in the responding tissue (Sokol and Melton, 1991).

The most important question, upon analysing the potential roles of these peptide growth factor-like molecules as natural mesoderm inducers, is whether their temporal, spatial and amount of expression is in accordance with mesoderm formation in the developing Xenopus embryo. It seems that the activins are the most likely candidates for playing this role in mesoderm induction, but the involvement of several other members of both the TGF-family and FGF family have been proposed.

Rebagliati et al., (1985) showed by differential screening of a cDNA library that most maternal mRNAs are uniformly distributed along the animal-vegetal axis, but a rare class of mRNAs are localised. Localised mRNAs may serve as cytoplasmic determinants by coding for proteins that specify cell types or at least create a morphogen that will act on other proteins in the specification of tissues. For example, in situ hybridisations show that Vq-1 (a member of the TGF- β family) mRNA (Weeks and Melton, 1987) is localised in the cortical shell at the vegetal pole of

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oocytes and in unfertilised eggs the hybridisation signal broadens as it spreads toward the animal pole. This movement of Vg-1 mRNA towards the equator ensures its distribution, during cleavage, to most vegetal cells and therefore the equator cells that will form mesoderm lie adjacent to cells containing Vg-1 RNA. The 2.3kb cDNA of Vg-1 produces a protein of 41.8kD which has a signal sequence possibly allowing its insertion into the endoplasmic reticulum (ER) for subsequent secretion (although natural secretion has never been detected), suggesting, together with its homology to TGF- β and its localisation, that Vg-1 represents a strong candidate for a natural MIF (Weeks and Melton, 1987). TGF- β 1 has no mesoderm inducing activity although it can enhance the level of induction produced by bFGF, so Vg-1 (a TGF- β related protein) could act with FGF in the synergistic induction of mesoderm (Kimelman and Kirschner, 1987). However, no in vitro mesoderm inducing activity has been reported for Vg-1 alone or in combination with other MIFs, so this model remains purely hypothetical.

Xenopus bFGF as well as bovine aFGF and bFGF and two proto-oncogenes related to FGF, kFGF and int-2, possess mesoderm-inducing activity (Paterno et al., 1989). Interest in these growth factors was revived by the finding that basic FGF is expressed maternally and is present at the time of mesoderm induction in the early Xenopus embryo. Furthermore, both the protein and the mRNA for the FGF

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receptor are known to be present in the embryo (Kimelman et al., 1988; Gillespie et al., 1989; Musci et al., 1990). The occurrence of FGF receptors, judged by binding of FGF in early embryos, follows a temporal pattern that is consistent with the period of competence during which animal pole cells are induced to become mesoderm by FGF (Friesel and Dawid, 1991). The FGF receptors so far identified in Xenopus embryos share similarities with other FGF receptors in regions such as the signal peptide, a conserved stretch of acidic residues in the extracellular domain, and a tyrosine kinase domain. The expression of these XFGF receptor mRNAs occurs throughout early development and is regulated by growth factors in animal cap explants. The activation of the tyrosine kinase activity by ligands such as FGF may therefore play a role in mesoderm induction, tyrosine phosphorylation forming part of the signalling mechanism of the induction (Friesel and Dawid, 1991). A dominant negative mutant of the FGF receptor has recently been shown to disrupt mesoderm formation in Xenopus embryos by inhibiting the FGF signalling system (Amaya et al., 1991). This truncated form of the FGF receptor, a construct referred to as XFD, encodes the complete extracellular domain and the transmembrane domain, but lacks all but seven amino acids of the intracellular tyrosine kinase domain. After ectopic expression as a result of micro-injection of synthetic XFD message, the ability of explanted animal caps to respond to

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exogenous FGF was assayed. Blocking of endogenous FGF receptors by XFD was monitored by observing the changes that occurred in the explant morphology, and by determining the level of muscle-specific cardiac actin in individual animal caps using an RNAase protection assay. Animal caps isolated from XFD embryos showed a much reduced morphological response to FGF than the control embryos and expressed a much lower level of muscle actin mRNA. Furthermore, whole embryos developed abnormally from the midgastrula stage (after injection with XFD message). Gastrulation was disrupted, resulting in specific aberrations in the formation of lateral and posterior mesoderm. However, overexpression of a wild-type FGF receptor could correct these defects suggesting that the mutant receptor specifically disrupts the FGF signalling system (Amaya et al., 1991). Results, however, indicate that FGF is only partially responsible for posterior mesoderm formation.

Experiments carried out using the peptide growth factors (PGFs) described above show that different PGFs induce mesoderm with different dorsoventral character. For example, members of the TGF- β family induce more dorsal mesodermal cell types than FGF-related factors (Ruiz i Altaba and Melton, 1989a; Ruiz i Altaba and Melton, 1990). Furthermore, as described above for XTC-MIF, the concentration of PGF affects the amount and type of dorsoventral tissue induced. The embryonic equivalents of

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FGF, TGF- β , activin B and XTC-MIF (activin A) may act, therefore, as mesoderm inducers and as dorsoventral patterning signals and morphogens. The dorsoventral differences could be due to the differential activation of PGFs involved in mesoderm induction on one side of the egg; for example, the secretion of FGF or the translation of activin RNA could be graded along the dorsoventral axis. Furthermore, it is clear that further secondary interactions such as the 'dorsalising' signal (signal 3 in the model; figure 1) of dorsal mesoderm on more ventral mesoderm are required to elaborate dorsoventral patterning. At present, activin is thought to be the dorsal inducer (signal 1) and FGF the ventral inducer (signal 2); whether or not this is the case, it seems certain that the activins and the FGF family of growth factors do play a crucial, specific role in Xenopus embryonic induction.

The criteria for establishing whether mesoderm induction has taken place in these isolation and recombination experiments has been based on histological differentiation of cells, characteristically shaped multicellular arrangements and most recently by the presence of molecular markers. Responses to mesoderm induction can be monitored by the rapid changes in gene expression. For example, genes turned on when the genome is transcriptionally activated at the mid-blastula transition (MBT) about 8 hours after fertilisation (Newport and Kirschner, 1982a and 1982b), such as mix.1 (Rosa, 1989), an endodermally-expressed

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homeobox gene sharing 56% homology to the Drosophila 'paired' homeobox, provide early markers of mesoderm induction. MyoD, an important regulatory molecule in myogenesis, is expressed in the future somite region and is an earlier marker than α -actin; however, it is also expressed at MBT (Hopwood et al., 1989). Non-muscle parts of mesoderm (and cephalic neural crest) express a gene called Xtwi - a Xenopus homologue of the Drosophila gene twist that is involved in mesoderm formation. Recently, a Xenopus wnt-1/int-1 related gene which is responsive to MIFs has been isolated (Christians et al., 1991). Xwnt8 may play a role in ventral mesodermal patterning during embryogenesis; like other wnt-1 related proteins its structure indicates it is a secreted molecule with a role in cell-cell signalling (Wilkinson et al., 1987). Xwnt8 is expressed in response to mesoderm induction by peptide growth factors and has a putative role in decoding and transmitting positional information within the embryo, that is, acting as a secondary signal in mesoderm induction. The temporal expression of Xwnt8 coincides with the time when newly induced mesoderm is supplied with positional information for establishing the major body axes. These four genes provide early molecular markers of tissue type and germ layer, but may also play a role in controlling the level of MIFs in early embryogenesis.

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1.1.5 Gastrulation and the establishment of the anterior-posterior axis

Gastrulation, the first and most major morphogenetic movement of tissues in embryogenesis, involves tissues of the outer layers of the embryo invaginating at the blastopore in an active and coordinated manner. As a result of gastrulation, the hollow ball of the blastula is transformed into a multilayered gastrula which has a new cavity, the archenteron, while the blastocoel is severely reduced in size. Gastrulation establishes new arrangements of the germ layers and lays out the blueprint for the basic bodyplan of the tadpole (Gerhart and Keller, 1986).

The morphogenetic processes that take place in gastrulation are coordinated in the intact embryo, but can occur independently according to a predetermined pathway until they are redirected by other tissues. The cellularised vegetal base induces nearby animal hemisphere cells to become extending marginal zone cells, of which the closest to the most inductive region of the vegetal base, begin their convergent extension first and most vigorously compared to those at greater distances from this region. Bottle cells - epithelial cells of the animal moiety located at the surface of the vegetal base - invaginate and subsequently migrate to the blastocoel roof. The spreading migrating deep zone cells are the most strongly induced because of their close proximity to the vegetal core (Gerhart and Keller, 1986). Nieuwkoop first demonstrated

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(1973) that the vegetal base of the midblastula induces adjacent animal hemisphere cells to engage in marginal zone activities, leading to the formation of mesodermal and endodermal structures (endo-mesoderm induction). The inductions cause marginal zone cells to undergo convergent extension, spreading migration and bottle cell formation instead of epiboly; the vegetal cells also induce dorsoventral differences in intensity and time of onset of cellular activity in the marginal zone (Gerhart and Keller, 1986). Convergent extension is an autonomous activity of the marginal zone and is the major regional activity during gastrulation as a whole. It drives involution of the marginal zone, closure of the blastopore, elongation of the anterior-posterior axis and accumulation of cells on the dorsal midline.

The cells of the marginal zone cannot initiate gastrulation activities until gene expression has started: α -amanitin-inhibited embryos do not gastrulate even though they develop to the late blastula stage (Newport and Kirschner, 1982b). Zygotic gene expression is therefore required to replace some maternal materials exhausted in the pregastrula stages or to provide new molecules, but the initiation of gastrulation is also dependent upon completion of events such as the recruitment of mRNAs, such as histone H1, into polysomes (Woodland et al., 1979).

As a result of gastrulation movements the dorsal axial mesoderm comes to lie immediately beneath the ectoderm

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which it induces to form neural and epidermal structures. Dorsal cells that migrate the furthest will become anterior and those that migrate the least will be posterior. The different AP positional values acquired by mesodermal cells can be revealed by their ability to induce different neural and epidermal structures. Grafting experiments where different regions of mesoderm are grafted into the blastocoel of a host embryo have demonstrated, for instance, that more anterior mesoderm induces more anterior neural or epidermal structures. If mesoderm migration is arrested by treatments such as brief cold shock (2°C, 4 min.) in the first cell cycle, or ultraviolet (UV) irradiation of the vegetal surface at the same stage, the prospective anterior mesoderm cells stop midway and differentiate into trunk structures. The inhibited egg develops into a headless, cylindrically symmetric embryo that lacks a vertebrate body axis (Gerhart et al., 1984).

UV irradiation of the vegetal pole of a fertilised egg causes a delay in gastrulation and produces embryos with a dose-dependent graded series of anterior deficiencies, very high doses causing embryos to exhibit dorsal as well as anterior deficiencies (Vincent and Gerhart, 1987 and references therein). Lithium treatment produces almost the opposite effect resulting in a dose-dependent graded series of posterior-deficient embryos (Kao et al., 1986). At moderate doses of lithium, applied at the early blastula stage, embryos develop with posterior defects in the tail

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and trunk so that only the head forms properly. Embryos ventralised by UV irradiation can be rescued by lithium treatment, suggesting that the latter effect is mimicking an event occurring at the multicellular stage. One major biochemical effect of Li is to block the resynthesis of phosphatidyl inositol biphosphate (PIP_2) by inhibition of the enzyme inositol monophosphatase (normally responsible for regenerating free inositol from inositol triphosphate (IP_3) and diacylglycerol (DAG)). The inhibition of this cycle results in reduced levels of IP_3 and DAG and since the effect of Li can be inhibited by co-injection of myo-inositol (Busa and Gimlich, 1989), may imply a role for the cycle in mesoderm formation and the establishment of AP fates; perhaps the dorsal side of the embryo has a depressed cycle and the ventral side an active cycle (Slack, 1991). In addition to the observations after subjecting blastulae to lithium, evidence favouring a role for a secondary message includes injection of embryos with polyoma middle T mRNA. Lithium increases the sensitivity of animal cells to both XTC-MIF and FGF, although it is unknown whether lithium is affecting the induction response or has later effects on dorsalisation. Intracellular signalling pathways may be activated during the natural inductive process since micro-injection of the viral oncogene polyoma middle T mRNA into early embryo results in the respecification of isolated prospective ectoderm to form characteristic mesodermal structures (Whitman and

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Melton, 1989b). Polyoma middle T is localised to the plasma membrane where it associates with and activates the cellular tyrosine kinase pp60^{C-src}, pp62^{C-yes} and a cellular phosphatidyl-inositol(3)kinase. These associations are necessary for the oncogenicity of polyoma middle T and its ability to induce mesoderm; mutants of middle T that cannot form these associations are non-transforming and incapable of inducing mesoderm.

1.1.6 Involvement of homeobox genes in mesodermal patterning.

Vertebrate homeodomain proteins are transcription factors with a DNA binding domain of 60 amino acids that is conserved in many different proteins (reviewed in Wright et al., 1989a and Gehring et al., 1990). In Drosophila, the expression of different homeobox genes in bands specifies segment identity along the AP axis of the early embryo (Gehring, 1987). Vertebrate homeodomain proteins may have an analogous role in embryogenesis; for example, XlHbox1 protein is expressed in the nuclei of cells that form across the somitic, intermediate and lateral plate mesoderm, the central nervous system and neural crest cells, with the AP borders in all of these cells aligned (Wright et al., 1989b). The distribution and function of this and some other Xenopus homeobox genes is summarised in table 3).

Genetic evidence that vertebrate homeobox genes are

Table 3.

Expression and function of some *Xenopus* homeobox genes.

1. Cho *et al.*, (1987). *EMBO J.* 7: 2139-2149.
2. Muller *et al.*, (1984). *Cell* 39: 157-162.
3. Cho *et al.*, (1991). *Cell* 65: 55-64.
4. Wright *et al.*, (1988). *Development* 104: 787-794.
5. Condie and Harland (1987). *Development* 101: 93-105.
6. Ruiz i Altaba and Melton (1989).
7. Harvey *et al.*, (1986). *EMBO J.* 5: 1237-1244.
8. Wright *et al.*, (1987). *EMBO J.* 6: 4083-4094.
9. Blumberg *et al.*, (1991). *Science* 253: 194-196.
10. See 9.
11. See 9.
12. See 9.
13. See 9.
14. See 9.

Name	Class of homeobox gene most related to	Region and timing of expression	Proposed function
1. Xlhbox1 (Xeb-1) (AC-1) (homologue of mouse Hox 3.3)	Antp	Narrow band across cervical region of CNS, neural crest and mesoderm. 2 proteins are produced from same ORF, 'long' and 'short' protein. Short protein expressed more anteriorly than long protein in mesoderm and CNS. A 2.3kb transcript is first detected at late gastrula, but major 1.6kb transcript is present at all early embryonic stages.	Activated more by XTC-MIF than by bFGF. Overexpression of short protein and injection of anti-long protein antibody result in similar asymmetries in the CNS and malformations that resemble hind brain. Role in correct morphogenesis of anterior spinal cord, that is dependent on their ability to recognise specific DNA sequences.
2. Xlhbox2 (MM-3)	Antp	2.1kb transcript expressed during oogenesis; almost disappears by blastula stage and then remains present until tadpole stage.	Maternally-expressed gene, therefore could have very early role in pattern formation
3. Xlhbox6	Antp	First transcribed at late gastrula /early neurula. 5x increase in level of transcripts between st. 13-20. Gradual spatially restricted expression along AP axis, until solely expressed in posterior neural cells.	Preferentially activated by bFGF in cultured animal cap fragments. Transplant experiments with animal caps, injected with Xlhbox6, suggests tail organiser activity; may be component of biochemical pathway generating AP axis.
4. Xlhbox8	Antp	Nuclear protein first appears in narrow band of endoderm at st. 33. As development proceeds, it becomes restricted to nucleus of endodermal cells of duodenum and developing pancreas, i.e. posterior parts of the foregut.	Restricted to AP band of exclusively endoderm, i.e. specifies positional information in endoderm. Involved in earliest specification of epithelial identity. Perhaps a target for an epithelial instruction signal from the mesoderm.

Name	Class of homeobox gene most related to	Region and timing of expression	Proposed function
9. Goosecoid	Gooseberry (51% identical)	1.3kb mRNA starts to get expressed at stage 8.5, peaks at stage 10.5 and decreases a lot by stage 13 (beginning of gastrulation). Localised to dorsal tip region.	Functional similarity to <u>bicoid</u> on the basis that it binds to the same DNA target; i.e. involvement in establishing AP polarity. Temporal expression pattern similar to Mix-1; expression also primary response to growth factor treatment.
10. Xlab	Labial Hox 2.9 Hox 1.6 Glox lab	First detected t stage 10.5, peaks at mid gastrula (St. 11.5)	Xhox lab genes probably involved in activation of posterior-specific genes and repress expression of anterior-specific genes. Mouse and chicken homologues are expressed in ectoderm/mesoderm of posterior of embryos. Overexpression in transplanted animal caps leads to secondary inductions, eg. tail-like structures and somite and neural inductions located anteriorly and posteriorly, and proximally to secondary axes.
11. Xhoxlab-1		RNA found in middle and head regions. Retinoic acid induces expression of this gene in retinoic acid - sensitive head region	
12. Xhoxlab-2		RNA in middle region. Retinoic acid treatment causes localised expression in head and tail regions.	
13. Xcad1	mouse <u>cdx</u> gene	First detected at stage 10.5, reaches peak at stage 13 and decreases to low amounts at stage 15	Perhaps activated by goosecoid gene product and involved in further refinement of AP axis.
14. Xcad2	Caudal-related gene class.	Enriched in dorsal lip region.	

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involved in specifying positional information has been provided, in the case of amphibians, by disrupting their function by embryonic manipulations. Injection of synthetic message (Harvey and Melton, 1988) or antibodies (Cho et al., 1989; Wright et al., 1989b) corresponding to two different homeobox genes (Xhox1-A and Xlhbox1) causes gross malformations in the somites or deletion of neural crest derivatives respectively. Embryos injected with homeobox genes are affected by an alteration in their pattern formation rather than their cell differentiation. For example, the use of molecular markers and histology have shown that injection of Xhox-1A consistently resulted in only somite defects suggesting a role for this gene in correct somite formation (since the phenotype occurs where this gene is normally expressed; Harvey and Melton, 1988). Xlhox6 over-expression can confer axis-organising activity; the secondary axes formed are not entirely normal and less complete than those induced in the presence of XTC-MIF (Cho et al., 1991). This ability to induce uncommitted cells to form part of a secondary axes is equivalent to organiser activity; perhaps Xlhbox6 inductions could represent tail organiser activity.

A recent screening of a Xenopus dorsal lip cDNA library with a degenerate oligonucleotide that recognises different homeobox genes led to the identification of four types of cDNAs that are specifically expressed in the organiser (Blumberg et al., 1991). The most abundant of these was

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goosecoid, which contains a homeobox which is 57% homologous to that of gooseberry and 32% to the bicoid homeobox. The goosecoid homeobox is similar (53%) to the homeobox of mix-1, whose expression, as described previously, is in direct response to mesoderm induction. The 1.3kb goosecoid mRNA is first expressed in the organiser at stage 8.5 and peaks at stage 10.5 which suggests, bearing in mind its location and functional similarity to bicoid (the goosecoid protein binds preferentially to an oligonucleotide containing a bicoid-binding site) that goosecoid may play a role in setting up axis formation. Perhaps overexpression of goosecoid mRNA may lead to uncommitted embryonic cells acquiring axis-forming activity, as is the case for Xlhbox6 (Cho *et al.*, 1991). The expression of homeobox genes, therefore, is likely to be a fundamental step in setting up axis formation and activation of downstream genes (including homeobox genes) may further refine the axis.

, Xhox3 is one of the zygotic genes that are first expressed at MBT, about 8 hours after fertilisation (Ruiz i Altaba and Melton, 1989b). Xhox3 RNA is expressed in a graded fashion along the AP axis in the axial mesoderm; lowest levels are at the anterior end and highest levels at the posterior end. In XTC-MIF or bFGF-induced mesoderm Xhox3 expression is easily detected by the early gastrula stage and is therefore an early response to mesoderm induction (injection of Xhox3 mRNA into animal caps does

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not result in mesoderm induction). Homeobox genes in other organisms are often expressed in response to inducing agents, representing activation of specific transcriptional programmes in response to a change of cell fate (Ruiz i Altaba and Melton, 1989a; Ruiz i Altaba and Melton, 1990). Over-expression of Xhox3 mRNA in the anterior end of normal embryos results in a failure to form normal anterior structures, thus the amount of Xhox3 mRNA marks the positional value of mesoderm. However, changing this level of RNA is not sufficient to change its AP character because the tissue that does not form anterior structures becomes undifferentiated rather than forming posterior structures (that is, a homeotic change does not occur). Xhox3 expression is enhanced fivefold in UV-treated embryos and fivefold depressed in lithium-treated anteriorised embryos (Ruiz i Altaba and Melton, 1989b).

Different levels of Xhox3 expression and therefore AP pattern are determined by different peptide growth factors (PGFs) (Ruiz i Altaba, 1989a). High doses of bFGF administered to isolated animal caps result in the formation of dorsal/posterior mesoderm and high levels of Xhox3 mRNA; high doses of XTC-MIF results in dorsal/anterior mesoderm and low levels of Xhox3 mRNA. Different peptide growth factors therefore induce mesoderm with different AP character as judged by the level of Xhox3 expression and the character of secondary neural/epidermal structures that are induced by this mesoderm (when

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implanted as observed in Mangold's grafts). The peptide growth factors can therefore act as morphogens that pattern the AP axis in addition to the DV axis. The establishment of AP polarity requires a system of positional information that specifies different cell fates for mesodermal cells according to their position at the end of gastrulation. This system may induce a diffusible graded signal consisting of one or more PGFs. Mesodermal cells may interpret the positional information set up by these gradients by measuring the types and dose of PGF and setting the level of homeobox gene expression accordingly; the 'organiser' could serve as a source for the gradient (Ruiz i Altaba and Melton, 1989a; Ruiz i Altaba and Melton, 1990). Other homeobox genes, including Xhox36 and XlHbox1 (see table 3), are also involved in the region-specific determination of cell fates (reviewed in Ruiz i Altaba and Melton, 1990).

Since the discovery of the conservation of the homeobox between flies and frogs (Carrasco et al., 1984), the low stringency hybridisations of Drosophila probes to Xenopus libraries has identified Xenopus relatives of Drosophila developmental regulatory genes. This technique has side-stepped some of the disadvantages of using Xenopus as an experimental animal such as the impracticability of screening for mutants, but leaves gaps in establishing causality which mutants provide.

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1.2 Dorso-ventral axis formation in Drosophila.

1.2.1 Brief Outline of Early Drosophila Development.

Drosophila, unlike Xenopus, undergoes merostic oogenesis, where 15 polyploid nurse cells pour their synthetic products into the oocyte via open cytoplasmic channels, called ring canals (reviewed in De Pomerai, 1990; Scott and O'Farrell, 1986). The nurse cell cluster is joined to the future anterior pole of the oocyte, which occupies a terminal position within the egg chamber. The nurse cell/oocyte complex is enclosed by a layer of follicle cells; polytenisation of the chromosomes and selective amplification of the chorion protein genes occurs in this complex later in oogenesis. During oogenesis, the nurse cells synthesise a variety of maternal RNAs and proteins, including determinants for DV and AP polarity, which are transferred to the oocyte. The Drosophila egg consists of a central nucleus surrounded concentrically by cytoplasm, a thick layer of yolk, a thin shell of periplasm, and the outer chorion sheath. After fertilisation - accomplished by a single sperm penetrating through the chorion via the micropyle - the zygote nucleus undergoes eight synchronous divisions without cytokinesis. During the 7th-8th cleavage most of the nuclei migrate towards the periphery of the egg where at the 12th cleavage, they form a single syncytial layer (syncytial blastoderm stage). At cellular blastoderm (14th cleavage)

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cell walls begin to form between nuclei, although the basal end remains open to the yolk mass for a while. Gastrulation follows, at which time the mesoderm invaginates ventrally, anterior and posterior mid gut invaginations form endoderm and pole cells come to lie within the posterior mid gut; cell walls now enclose most nuclei. The posterior mid gut moves dorsally and forwards, extending the germ band which subsequently becomes divided into metameric units. Position along the DV axis will primarily determine whether cells within the germ band will become mesoderm, ventral neuroblasts/epidermis or dorsal epidermis. Once the metameric subdivision of the germ band is established, the germ band shortens again and the embryo hatches into a first instar larva.

The hatching Drosophila larva exhibits a highly organised segmented pattern of differentiated structures, which is in contrast to the homogeneous appearance of the fertilised egg. However, the fertilised egg has an intrinsic polarity which promotes spatial cues for the determination of the DV and AP axes. The establishment of these primary axes in Drosophila is directed by maternal-effect genes, that is, genes for which the phenotype of the embryo depends upon the genotype of its mother (Nusslein-Volhard, 1991). Three independent systems of maternal-effect genes along the AP axis determine the analgen of the anterior (head and thorax), posterior (abdomen) and terminal (acron and telson) elements of the larva (Nusslein-Volhard et al.,

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1987). In contrast, a single system of positional information is required for the determination of pattern elements along the DV axis. These pattern elements consist from ventral to dorsal of: mesoderm formed from the the most ventral strip of cells which invaginate during gastrulation; ventral epidermis and ventral neurogenic region; dorsal epidermis; the amnioserosa (an extra-embryonic structure formed from the most dorsal strip of cells). Thus the AP pattern is a repetition of several metameric units whereas the DV pattern is a non-repetitive series of cell types (Anderson, 1987). Mutations that disrupt maternal cues in the DV axis do not, in general, affect those in the AP axis and vice versa (Nusslein-Volhard et al., 1987). It is known that three of the four systems (i.e. anterior, posterior, terminal and DV systems) are known to depend upon the asymmetric distribution of gene products in the egg; bicoid (anterior), nanos (posterior) and a nucleocytoplasmic gradient of dorsal(DV).

- 1.2.2 Dorsal-Ventral (DV) Polarity of the Egg and Embryo.

DV polarity resembles more closely the terminal system, rather than the anterior and posterior systems of AP axis determination. In the latter two systems, the signal, represented by localised mRNA, occurs within the germ-line derived oocyte-nurse cell complex. However, the terminal and DV systems require contact between follicle cells and the oocyte (reminiscent of inductive interactions in

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Xenopus) to create the polarity of the egg (reviewed by Nusslein-Volhard, 1991).

Maternally-active genes that initiate DV polarity fall into two groups: 'early acting genes', that determine DV polarity of the oocyte within the egg chamber and; 'late acting genes', which determine DV polarity of the embryo following fertilisation (Anderson et al., 1985a; figure 3). Three 'early acting' genes are involved in the establishment of DV polarity in both the egg and embryo. Fs(1)kl0, which encodes a protein with a helix-turn-helix motif, is thought to have a regulatory function in establishing the intrinsic polarity of the oocyte; fs(1)kl0⁻ mutants give rise to dorsalised embryos (Prost et al., 1988). Mutations in torpedo and gurken genes, which are derived from the somatic and germ-line components respectively, give rise to ventralised embryos. Somatic and germ-line cells in this case, cooperate during oogenesis to establish the normal DV polarity of the egg and embryo; Interestingly, torpedo⁺ product shares homology with the mammalian EGF receptor, suggesting a role in cell communication, where the soma may receive a signal from the oocyte. Most other maternal-effect genes act autonomously within the germline and mutants display the phenotype, even in the presence of the wild-type follicle cells; exceptions include nudel, pipe and windbeutel, which act in the soma.

The 'late acting genes' comprise 12 genes (figure 3) including cactus and 11 'dorsal group' genes - dorsal(dl),

Figure 3.

Schematic representation of the dorsoventral polarity pathway in Drosophila leading to the formation of a nuclear gradient of dorsal protein.

Mutations in the genes which are underlined give rise to ventralised embryos. Mutations in all other genes give rise to dorsalised embryos.

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easter(ea), gastrulation defective(qd), nudel(ndl),
pipe(pip), pelle(pll), snake(snk), spatzle(spz), Toll(Tl),
tube(tb) and windbeutel(wind) - that result in almost
identical phenotypes when inactivated. Null mutations in
any one of the 'dorsal group' genes causes 'dorsalisation'
of the embryo, where embryonic cells in both dorsal and
ventral regions follow a pathway of dorsal differentiation
(Anderson, 1987). This common phenotype indicates that a
cascade of interactions among 'dorsal group' gene products
may specify a morphogen gradient, which ascribes unique
positional information to each cell along the DV axis
(Anderson et al., 1985a). Maternal mutants that cause
ventralisation are as a result of dominant mutations at
loci whose null (recessive) phenotypes are dorsalisied (eg.
Toll^D), except for cactus, a maternally acting null mutant
which causes ventralisation of the embryo. The wild type
functions of the 'dorsal group' genes, therefore, are
required to establish ventral pattern and the dorsal
pathway may arise as a default state when one or more of
their products are deficient (Anderson, 1987).

Experiments involving 'dorsal group' genes have been
carried out to determine the distinct roles of the
individual genes during DV pattern formation and to try to
establish the functional hierarchy of the pathway. Some
recovery of DV pattern is observed following injection of
wild-type (wt) cytoplasm into the mutant embryos of several
'dorsal group' genes (Santamaria and Nusslein-Volhard,

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1983; Anderson, 1985a). For most of the 'dorsal group' genes, where cytoplasmic injection results in rescue (dl, tb, ea, snk, pll and spa), the polarity of the restored pattern is normal and corresponds to the original polarity of the asymmetric egg, rather than the site of injection, implying that these mutant phenotypes retain an intrinsic DV polarity. The polarity of Toll⁻ embryos, however, is determined by the site of injection of the rescuing activity (Anderson et al., 1985b) and therefore these mutants lose the intrinsic polarity of the egg. The Toll gene is therefore uniquely important in the differentiation of DV pattern since it is the only gene whose product is required for both the formation of ventral pattern elements and for the definition of the polarity of the DV axis. It seems that Toll product is present in an inactive state on the dorsal side of embryos since 'dorsal' cytoplasm does not induce ventral tissue formation in situ, but when injected it can rescue Toll⁻ embryos. The rescuing activity must, therefore, be a precursor form of the active Toll product (Anderson et al., 1985b).

The importance of Toll in the establishment of DV pattern formation is further supported by the existence of dominant gain-in-function mutants at this locus. Dominant regulatory mutations result in the constitutive and ectopic expression of genes in cells where their activity is inappropriate. Embryos that are derived from mothers that are heterozygous for any one of several Toll^D alleles (eg. Toll^{9Q} and Toll

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10b) are ventralised, that is, their dorsal regions follow a more ventral pathway of development. This ventralised phenotype could be due to an altered Toll protein which is indiscriminately activated on both the dorsal and ventral regions of mutant embryos (Anderson et al., 1985b). Double mutant crosses indicate that Toll acts downstream of all other 'dorsal group' genes except dorsal, tube and pelle.

The Toll gene product is a transmembrane protein - suggesting that it does not control DV polarity by directly modulating gene expression - with a cytoplasmic domain of 269 aa and an extracytoplasmic domain of 803 aa including an N-terminal signal sequence (Hashimoto et al., 1988). Sequence similarity of the cytoplasmic domain of Toll product to the cytoplasmic domain of the interleukin 1 receptor (IL-1R) and the mapping of the loss of function Toll alleles to this same region suggests that these two proteins transmit their signals by similar mechanisms (Schneider et al., 1991). The extracytoplasmic domain contains multiple repeats of a leucine-rich sequence found in many proteins with diverse biological functions (Hashimoto et al., 1988; Krantz and Zipursky, 1990). These Leu-rich repeats are thought to promote protein-protein interaction so two Toll polypeptides could associate non-covalently in the same membrane and this dimerisation could be essential for Toll to function as a receptor (Hashimoto et al., 1991). Many of the gain-in-function mutants arise from mutations in this extracytoplasmic domain (Schneider

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et al., 1991) suggesting that transmembrane communication of an extracellular signal is involved in establishing DV polarity of the embryo.

Easter is the only other 'dorsal group' gene that mutates to give detectable dominant alleles (Chasan and Anderson, 1989). Dominant alleles of easter result in the expansion of ventrolateral and lateral derivatives at the expense of more dorsal structures and show that easter has a function in the control of the spatial distribution of these structures in addition to their formation. Easter and snake encode extracytoplasmic serine proteases (Chasan and Anderson, 1989; DeLotto and Spierer, 1986) with structural similarities to known serine proteases such as kallikreins and blood clotting factors. Since Toll encodes a membrane protein with an extracytoplasmic domain, snake, easter and Toll could function in the same subcellular compartment, suggesting that Toll could encode a substrate for the easter or snake protease. However, although all three proteins are active at the same time - syncytial blastoderm stage - the substrate specificities (i.e. properties of the binding site) of easter (and snake) argue against either protease cleaving and activating the other protease zymogen.

Recent experiments have shown that the plasma membrane localisation of the Toll protein is required for its role in transmembrane signalling from the extracellular perivitelline space to the cytoplasm (Hashimoto et al.,

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1991). Furthermore, transplantation experiments have shown that perivitelline fluid from the compartment surrounding the embryo of Toll⁻ mutants can restore DV pattern to embryos from mothers mutant for windbeutel, nudel and pipe (Stein et al., 1991). The perivitelline fluid may contain products that activate the Toll product because the positioning of this transplanted perivitelline fluid also determines the polarity of the restored DV axis and rescuing activity requires that the fluid is from sites where toll protein would normally be active. Further evidence that the Toll activators occur in the perivitelline fluid includes the observation that rescuing activity can only be detected in eggs that lack the Toll gene product, because a proportion (depending upon stoichiometry) of any proposed ligand for Toll will be bound in Toll⁺ embryos and unavailable for transplantation, whereas more of it will remain diffusible and transplantable in the fluid in Toll⁻ mutants.

Studies on double mutant combinations have concluded that Toll^D products can promote, to some extent, the formation of ventral structures in the absence of most other dorsalisating gene products, suggesting that most of the 'dorsal group' gene products appear to influence DV pattern indirectly, through modulation of Toll⁺ activity (Anderson, 1987). Dorsal⁻, Toll^D double mutants, however, display a fully dorsalisated phenotype that is indistinguishable from that of dorsal mutants, suggesting that the dorsal gene

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product, unlike other 'dorsal group' genes acts downstream of Toll.

1.2.3 Subcellular localisation of dorsal.

Cytoplasmic injection experiments indicate that cytoplasm taken from any region of a wt embryo just after fertilisation can rescue dorsal⁻ embryos, but dorsal⁺ gene activity (and therefore rescuing activity) becomes progressively enriched in ventral regions up to blastoderm stage embryos (Santamaria and Nusslein-Volhard, 1983). The dorsal⁻ embryos must therefore have an intrinsic polarity, which may be established by other 'dorsal group' genes, but the lack of dorsal product may prevent its expression in a visible form (Santamaria and Nusslein-Volhard, 1983). Genetic studies, using different dorsal alleles, reveal that the dorsal phenotype is sensitive to gene dosage. One of the last steps in the maternal gene cascade could therefore be the asymmetric distribution of dorsal⁺ product which could directly control DV cell identities. Support for this latter property has come from the finding that dorsal is a sequence-specific DNA-binding protein (Ip *et al.*, 1991; Thisse *et al.*, 1991).

The dorsal transcription unit is about 14kb and encodes a poly A⁺ RNA of about 2.8kb which is only found in oocytes, eggs and early embryos (Steward, 1987). Dorsal expression is restricted to the nurse cells of the ovary where it accumulates in a stable form in the maturing egg. Dorsal

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mRNA persists until about 2 hours after fertilisation and cannot be detected by cellular blastoderm formation. In situ hybridisation reveals that all dorsal mRNA is distributed uniformly throughout the ooplasm and cytoplasm of the early embryo (Steward, 1987), thus the asymmetric distribution of dorsal is not at the level of its RNA.

Recent experiments have indicated that the asymmetric distribution of dorsal⁺ rescuing activity is due to a nucleocytoplasmic gradient of dorsal protein (Steward et al., 1988). The pathway of events leading to this gradient is shown in figure 3. Dorsal protein is distributed evenly in the egg at the time of fertilisation until cleavage stage. However, when the nuclei migrate to the surface of the embryo at syncytial blastoderm, the dorsal protein becomes localised to the nuclei and forms a ventral to dorsal gradient of nuclear localisation (Rushlow et al., 1989; Steward, 1989; Roth et al., 1989). Activated Toll⁺ product is thought to be directly involved in the nuclear uptake of dorsal protein. In addition to genetic studies and transplantation experiments, the gradient of nuclear localisation suggests that dorsal encodes a morphogen, that is, a product that is distributed in a gradient and determines positions along the axes in a concentration dependent manner.

The dorsal protein is present in the nuclei of those cells where its function is known to be required - ventral and ventrolateral regions - while in cells where it is not

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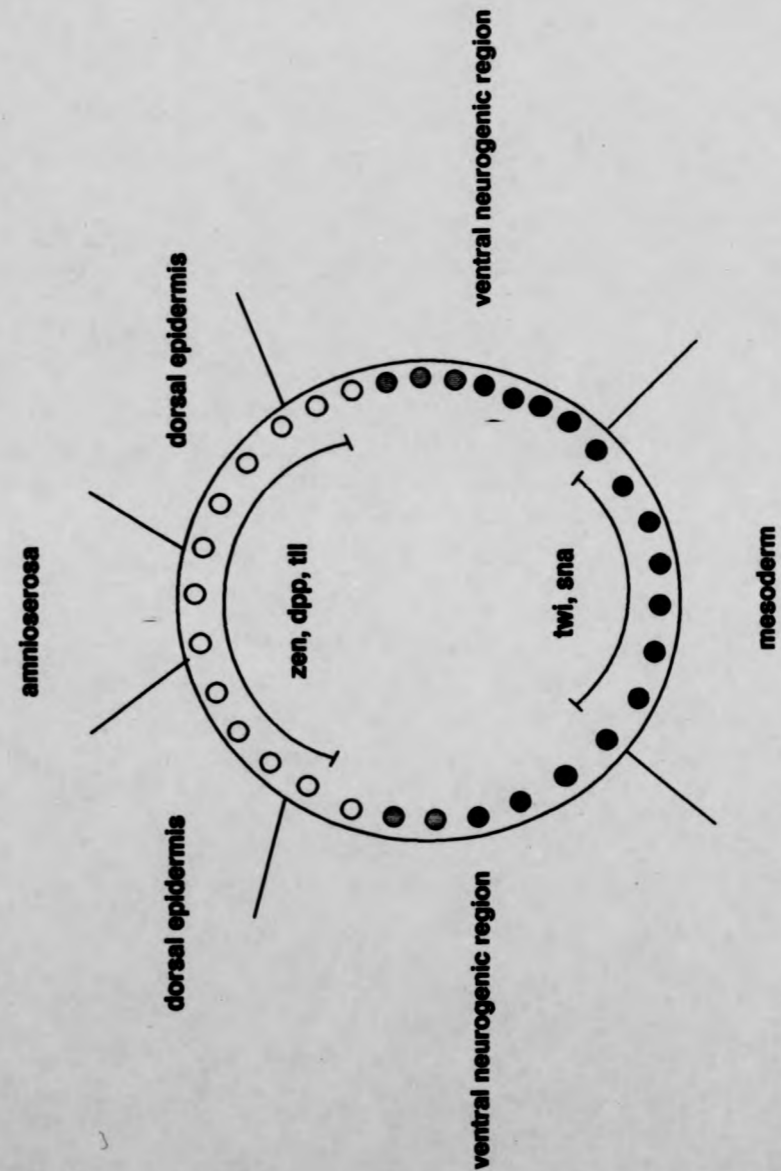
active it is restricted to the cytoplasm. Neither nuclear or cytoplasmic staining with anti-dorsal antibodies is observed in dorsal⁻ embryos (Rushlow et al., 1989). Maternal mutants genetically upstream of dorsal, when stained with anti-dorsal antibodies, show that although they contain high levels of dorsal protein, it is restricted to the cytoplasm and is not detectable in the nuclei. Nuclear staining is clearly observed in wild-type embryos by cleavage cycle 11. Three distinct domains of dorsal expression are observed along the DV axis: the presumptive mesoderm which contains peak nuclear levels; the lateral transition zone in the neurogenic region, which includes a steep gradient of nuclear expression - in a span of only 5-6 cells the nuclear level of dorsal protein falls from peak to little expression; and dorsal and dorsolateral regions, which contain little or no nuclear expression (figure 4). By stage 12,⁻ there is an overall gradient of dorsal protein since there is a general decline in levels of the protein in dorsal regions - probably as a result of selective degradation as a result of failure to be translocated to the nucleus; there is no RNA detected by cellular blastoderm, at Stage 14 (Steward et al., 1988). The protein exists until the onset of germband shortening (Rushlow et al., 1989).

Figure 4.

Schematic drawing of cross-section fate map of blastoderm stage embryo and initial expression pattern of zygotic genes.

Twist (*twi*) and snail (*sna*) are expressed in the presumptive mesoderm; tolloid (*tll*), zerknüllt (*zen*) and decapentaplegic (*dpp*) are expressed in the presumptive amnioserosa and dorsal ectoderm. (Adapted from Govind and Steward, 1991).

Circles represent nuclei of the cellular blastoderm. The black to white gradient of shading represents a high to low gradient of dorsal expression in these nuclei.



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1.2.4 Mechanism of transport of dorsal and the role of cactus.

The subcellular localisation of mutant and wt forms of the dorsal protein were studied in transfected Schneider tissue culture cells (Rushlow et al., 1989). In these cells, wt dorsal protein accumulated in the cytoplasm whereas truncated C-terminal mutants were translocated to the nucleus, suggesting that the C-terminal amino acids are required for cytoplasmic localisation of the wt protein. It is thought that in Schneider cells and the early embryo this cytoplasmic localisation signal may override the nuclear localisation signal (NLS : RRKRQK - similar to the NLS in SV40 large T antigen). Alternatively, the C-terminal residues may form a cytoplasmic anchor that retains dorsal in the cytoplasm by interaction with another protein - a situation analogous to the unliganded glucocorticoid receptor, which is associated via its C-terminal end to the hsp90 gene product until binding by the steroid allows its translocation to the nucleus (Picard and Yamamoto, 1987). A possible candidate for the role in preventing the translocation of the dorsal protein to the nucleus is the cactus gene product. Cactus is required in wt embryos for the specification of dorsal, dorsolateral and in part, ventrolateral structures (Roth et al., 1989). Double heterozygotes for cactus and most other 'dorsal group' genes develop into embryos that express ventrolateral pattern elements and show increased nuclear uptake of

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dorsal protein all along the DV axis. These embryos differentiate ventral epidermis around the entire egg circumference and in contrast to cactus embryos, they lack polarity and do not form a ventral furrow during gastrulation. Dorsal, however, is epistatic to cactus and the dorsalis phenotype is unaltered upon loss of cactus activity, suggesting that cactus acts via dorsal, as judged by cytoplasmic transfer. The cactus product is evenly distributed along the DV axis, implying that inhibition of nuclear transport of the dorsal protein caused by the cactus product is antagonised on the ventral side of the embryo (Roth et al., 1991) - probably as a result of the action of the 'dorsal group' gene products. The neomorphic gain-of-function alleles of cactus may produce a mutant cactus product that is still equally distributed, but cannot inhibit nuclear transport of dorsal protein. Perhaps 'dorsal group' gene products act on both cactus and dorsal gene products in order to prevent formation of a cytoplasmic complex and stimulate nuclear transport respectively.

1.2.5 The role of the dorsal protein and its interaction with zygotic genes.

As in the establishment of AP polarity, maternal DV gene products interact to generate a system of positional information in the early embryo, to which zygotic genes then respond with spatially restricted patterns of

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expression (Nusslein-Volhard, 1991). Zygotic genes are switched on or off in response to threshold amounts of maternal gene products. Thus, the maternal mutants affect the entire axis and the zygotic mutants have more limited effects on pattern, perhaps because their gene activities mediate the subdivision of pattern and are expressed in restricted areas (Anderson, 1987).

Twist and snail are zygotic genes, involved in DV pattern formation, that share the same phenotype; absence of the ventral furrow with the concomitant loss of mesodermal derivatives. Thus the wt products of twist and snail are required specifically for the ventral most regions that act as mesodermal precursors. However, twist transcripts are present in snail⁻ embryos, suggesting that they play independent roles in the development of mesoderm (Alberga et al., 1991). A close relationship between twist and dorsal has been established from genetic studies (Simpson, 1983). Double heterozygotes for twist and dorsal null alleles (dl^{-/+}; twi^{-/+}) show the same phenotype as for twist⁻ embryos, indicating that a reduced dose of dorsal product causes a lower activity of twist⁺. This effect is specific for dl^{-/+}; twi^{-/+} heterozygotes implying that twist does not interact with any other 'dorsal group' gene product. One of the ways dorsal may control DV polarity could be by initiating twist expression - which is only essential at gastrulation - at the ventral pole of the embryo. The twist product is present in the ventral most

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part of the embryo, at the same time and in the same region as the maximal nuclear concentration of dorsal protein (figure 4; Thisse et al., 1988). Twist RNA is not detected in mutant embryos derived from dorsal⁻ mothers (Thisse et al., 1987). Thus, the recent finding that dorsal binds specifically, in vitro, to twist promoter sequences (Thisse et al., 1991) was not entirely surprising (the consensus binding site is G/CAGAAANC/TC/G). Two regions of activation were located on the twist promoter, so it is possible that dorsal protein may bind cooperatively. Dorsal may also activate the expression of snail, a zinc-finger protein (Boulay et al., 1987), which is expressed in a region that undergoes a morphogenetic movement. Since the expression of snail ceases once movement is completed, it may have an indirect role in cell movement (Alberga et al., 1991).

Dorsal is also thought to influence DV fate by repressing zygotic gene expression. For instance, dorsal restricts the expression of zerknüllt (zen) (Rushlow et al., 1987) and decapentaplegic (dpp), a homologue of TGF- β whose action may also be mediated by a soluble growth factor-like molecule, (St. Johnston and Gelbart, 1987) to dorsal regions of the embryo, where they are required for differentiation of the amnioserosa and dorsal epidermis respectively (Wakimoto et al., 1984; Padgett et al., 1987). In dorsal⁻ embryos (or in embryos where dorsal fails to enter the nucleus) there is a failure to activate twist and snail, but zen and dpp are expressed in an unrestricted

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manner in both dorsal and ventral regions (Rushlow et al., 1987; Anderson, 1987). It was shown that dorsal specifically interacts with distal sequences of the zen promoter; the dorsal protein recognises a similar motif to that in the twist promoter (Ip et al., 1991). Three of the four binding sites present in the zen promoter map in a ventral repression (VR) element. This clustering of binding sites may be used in the sharp on/off initiation of zen expression pattern by a gradient of dorsal morphogen and may facilitate cooperative binding of the dorsal protein to the VR. A minimum threshold of dorsal protein may be required for repressing zen expression - perhaps all the sites need to be filled for 100% repression. Dorsal has a higher affinity (about 5 times) for the zen motif than the twi motif (Thisse et al., 1991). Since twist expression is only activated in the ventral region (corresponding to maximal nuclear concentration) while zen repression occurs even in territories in which the nuclear concentration of dorsal is low (dorso-lateral territories; figure 4) thereby restricting its expression to the most dorsal part of the embryo, dorsal must therefore be able to repress zen expression at nuclear concentrations that are insufficient to activate twist. Thus dorsal protein may act along the DV axis on different gene targets by specifically binding to low, moderate and high affinity sites from ventral to dorsal respectively.

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1.2.6 Analogy of DV axis formation in Drosophila to that in Xenopus.

In both Xenopus and Drosophila the polarity of the body axes is established by an initially even distribution of morphogenetic information that can autonomously generate a polarised state. In Xenopus, the DV axis can arise in any orientation and is normally fixed by the point of sperm entry (Gerhart et al., 1981). However, physical stimuli can override the effect of the SEP and redefine the polarity of the axis. The initial trigger must then be amplified by components homogeneously available within the egg to give rise to the definitive body axis. In Drosophila, the DV axis is established by a similar process. The hypothetical Toll precursor is distributed more-or-less evenly within the egg and normally responds to a localised trigger (contained in the perivitelline fluid) to become active on the curved, ventral side of the egg. Under certain appropriate conditions, Toll can become activated at other positions and thereby define a different polarity of the DV axis. One might speculate, therefore, that Xenopus homologues of Toll, dorsal, snail and twist may play roles that are similar to those played in Drosophila development. Whereas default states in Drosophila give rise to dorsalisated embryos, in Xenopus ventralised embryos result; this is expected since the axis of one is inverted with respect to the other.

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1.3 A Family of rel Proteins

As soon as it was sequenced, dorsal was found to have a high degree of identity, particularly in one extensive homology region in the N-terminal half which was shown to be 47% identical to the v-rel oncogene, the transforming component of the replication-deficient virus REV-T (reticuloendotheliosis virus strain T; Steward, 1987). This is an acute leukaemia virus of galliform birds. Both proteins are members of a growing family of rel-related proteins (see figure 5), which are a group of transcriptional regulators involved in cell differentiation. Proteins belonging to this family share a high degree of homology in their N-terminal halves, which are involved in DNA binding and dimerisation, while their C-terminal transcriptional activation domains are completely divergent. Rel family members are all translocated from the cytoplasm to the nucleus in response to a variety of extracellular signals.

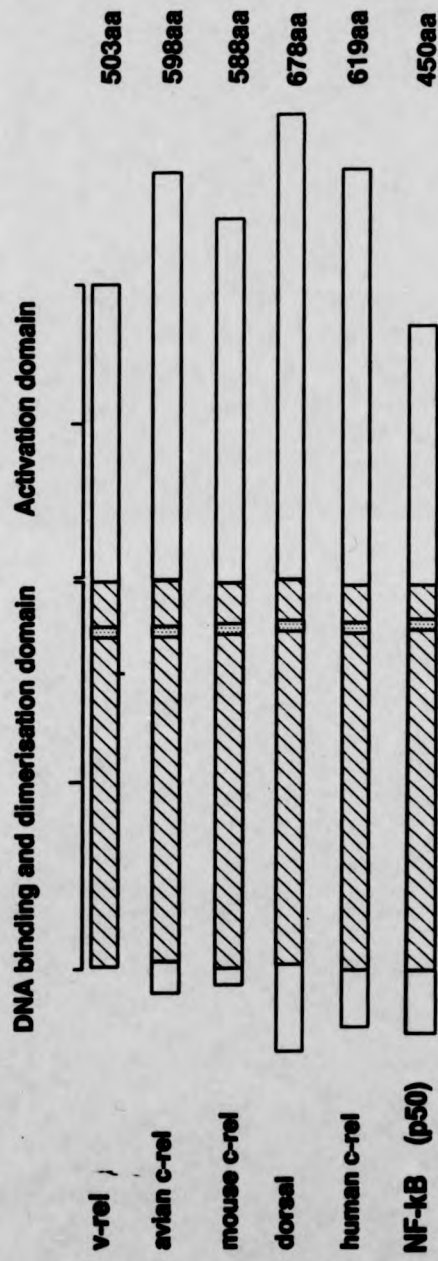
1.3.1 Structural characteristics of rel family proteins

The degree of similarity between N-terminal domains of rel family members (including x-rel protein; see Chapter 5) is shown later in Figure 14 and table 4. The region of all rel family members which has high similarity, the rel homologous (RH) domain, which also shares homology with the E. coli gene fnr (encoding a transcriptional regulator

Figure 5.

A family of Rel-related proteins.

Only some members of the rel family are shown here. NF-kB is represented by p50 in this diagram, but p65 is also related to c-rel. Proteins in this family share a high degree of homology in their N-terminal end, but are completely divergent in their C-terminal halves. The conserved region is thought to encode a DNA binding and dimerisation domain. The divergent region is thought to encode an activation region. Refer to figure 14 for Clustal sequence analysis between rel family members and to tables 4 and 5 for the % identity over the N-terminal region and over the full-length proteins.



- rep. nuclear localisation signal
- ▨ rep. 45-50% protein homology
- rep. completely divergent part of protein

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specific for genes involved in anaerobic transport; Chen et al., 1986), encodes a DNA binding domain that binds to common or related sequence motifs. Other families of transcription factors have been identified by virtue of their similar DNA binding domains, although the remaining regions of the proteins are unrelated. This has led to their classification into such distinct families as homeobox proteins, zinc-finger proteins, POU domain proteins and so on (reviewed in Gehring et al., 1990; Klug and Rhodes, 1987; Rosenfeld, 1991). The RH domain is involved both in dimerisation and DNA binding and these properties have been demonstrated in vitro for v-rel, p85 (human c-rel), murine c-rel (the mouse homologue of human c-rel), p50 and p65 of the NF-kB complex (Ballard et al., 1990; Inoue et al., 1991). Dorsal protein has also been shown to bind DNA in a sequence-specific manner, but dimerisation has not yet been demonstrated (Ip et al., 1991; Thisse et al., 1991).

All rel proteins contain a consensus nuclear localisation signal (NLS), which is present in the RH domain. Typically, the NLS consists of one stretch of basic amino acids (reviewed in Garica-Bustos et al., 1991), that have been shown in chicken c-rel, for example, to function as a NLS (Gilmore and Temin, 1988; Capobianco et al., 1990). Furthermore, a recognition sequence for phosphorylation of serine residues by cAMP-dependent protein kinase (Arg-Arg-X-Ser) is conserved near the NLS in all rel proteins,

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1.3.2 Functions of rel family proteins

As described in section 1.2.5, dorsal regulates embryonic DV polarity directly, by controlling the response of a set of target genes whose products are involved in creating the ventral axis. Dorsal protein was found to bind to common sequences present in the promoters of its target genes (for example, twist and zerknüllt). These sequences are similar to those recognised by NF- κ B in the κ light chain enhancer (and HIV-1 enhancer), and are known as the κ B element consisting of a decameric DNA sequence 5' GGGACTTCC 3', known as the κ B binding site.

Some research groups refer to the human transcription factors that bind to κ B sites collectively as NF- κ B (nuclear factor κ B). However, NF- κ B as originally identified as binding to the κ B binding site by Baltimore and co-workers, is now known to consist of a heteroligomer of p50-p65, and I will use this notation throughout the remaining text. Several other mammalian rel family/ κ B binding transcription factors have now been identified (p85 (p82^{hc}-rel), p75 and a cleavage product of p50 in humans, and mouse c-rel (probably the murine homologue of p85; see table 4).

NF- κ B is present in many cells in an inactive cytosolic form (reviewed in Baeuerle, 1991) in a wide variety of cell types, and under the influence of a variety of extracellular signals it becomes translocated to the nucleus, where it activates a range of genes (reviewed in

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Lenardo and Baltimore, 1989). The functions of NF-kB include immunoglobulin k chain transcription in B cells of the correct developmental stage; NF-kB is constitutively active and nuclear in these cells. NF-kB is also involved in T cell activation, which results in the concomitant activation of the interleukin-2 alpha subunit receptor (IL-2R α). NF-kB is induced in T cells by mitogens and is partly responsible for the increase in expression of the IL-2 receptor, which is required for T cell activation. IL-2 expression is also partly regulated by NF-kB. In HTLV-1 infected T cells, the viral protein, tax, induces NF-kB activity causing unregulated growth which may lead to leukaemia. In a monocytic cell line (U397), chronic HIV infection has been shown to induce NF-kB activity and permanently increase HIV enhancer activity (Bachelerie et al., 1991); the mechanism of induction in these cells is unknown, but appears to be involved with HIV replication. Proteolysis may regulate NF-kB activity since HIV-1 protease has been shown to process p105 (the precursor of p50) and thus increase levels of active nuclear NF-kB (Riviere et al., 1991).

In addition to IL-2, NF-kB is involved in the gene regulation of other cytokines (proteins conveying signals that regulate cellular proliferation and differentiation), such as IL-6, TNF- α , β -IFN and lymphotoxin. Finally, NF-kB is also involved in virus gene activation; it activates from kB binding sites in enhancers found in HTLV-1, HIV-1,

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SV40 and CMV viruses (reviewed in Lenardo and Baltimore, 1989; Baeuerle, 1991).

Due to its widespread distribution, there seems little doubt that NF-kB will be implicated in the regulation of many other genes. The specificity of NF-kB activity in particular cells is probably the result of a requirement for the interaction with other factors to activate a particular gene. The enhancers/promoters that contain kB binding sites consist of arrays of binding sites for other transacting factors, which may interact synergistically with NF-kB to elicit cell-type specific activation.

Since other human kB binding/rel proteins bind to the same sites as NF-kB, and are not generally cell-type specific, they have a potential role in the regulation of the same wide range of genes as NF-kB. However, the only clear evidence for a role for these proteins is in T cell activation (Molitor *et al.*, 1990). These authors suggest that T cell stimulation results in the rapid translocation of 55kd and 75kd rel proteins to the nucleus. The 55kd protein is p50 (a component of NF-kB), but p75 is distinct from the p65 subunit of NF-kB (Ballard *et al.*, 1990). The entry of two other rel proteins to the nucleus (50 and 85 kD proteins) is delayed. Proteolytic clipping indicates that the 50kD protein is a cleavage product of p50, and the 85kD protein is identical to p82^{hu} c-rel and HIVEN86A.

Within the rel family in other species, mouse and chicken c-rel are most similar to human c-rel (p85), at a level of

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80 and 78% amino acids respectively in the N-terminal region; however, whereas mouse and human c-rel are also similar in the C-terminal region, chicken c-rel is completely divergent. Chicken c-rel (and mouse c-rel) like NF- κ B, is also widespread, but is more abundant in cells of haemopoietic origin. Transcription of murine c-rel is inducible in fibroblasts in response to serum or TPA; mature lymphoid cells are the only cells in which constitutive expression of c-rel transcripts has been observed (Bull *et al.*, 1989). This is in contrast to NF- κ B, whose expression is widespread and induction is not at the level of transcription, but at the level of nuclear translocation. It is not known whether differential control of nuclear localisation operates with mouse c-rel. These data suggest a role for chicken and mouse c-rel in gene regulation in cells of lymphoid origin, although specific target genes are not known.

In general, expression of a proto-oncogene in a specific cell-type has not been found to correlate with the susceptibility of that cell to transformation by a virus carrying an activated form of that gene (reviewed in Bishop, 1991), but Rev-T transforms very immature B cells in vitro and in vivo and c-rel is expressed predominantly in the bursa - a lymphoid organ of birds associated with the lower gut - where B cell differentiation is thought to occur. V-rel could thus interfere with the normal transactivating function of c-rel, causing a rapid lymphoma

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in young galliform birds. However, although the principal 4kb transcript of avian c-rel is expressed predominantly in cells of haemopoietic origin, a smaller 2.6 kb transcript is expressed in either primary oocytes or developing follicle cells, as well as a low level of expression in haemopoietic cells. One could speculate that this 2.6kb transcript may have a functional role that is more similar to the role of dorsal. However, there is no sequence data reported for this gene and so it could be a more distantly rel-related family member.

1.3.3 DNA binding and transcriptional activation by rel family proteins.

As indicated in the previous section, the rel family in humans is so far known to consist of five proteins; p50, p65 (which together constitute NF-kB), p75, p85 (also known as p82^{huc-rel} and HIVEN 86A) and a cleavage product of p50 (known as p55, if the 50kD subunit of NF-kB is referred to as p55 as in the work published by Molitor and co-workers. (1990)). In other species, well studied examples of rel proteins include the dorsal protein in Drosophila, the c-rel proteins from chicken and mice and the v-rel protein, a deleted form of chicken c-rel, found in REV-T transformed lymphoid cells.

Rel proteins have been shown to act as transcription factors in co-transfection assays. V-rel - a truncated form of c-rel, lacking 2 N-terminal aa and 118 C-terminal aa

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(in addition to a few internal aa changes) - c-rel, v-rel/c-rel hybrids and dorsal can all activate transcription from a variety of promoters, including viral promoters such as the simian virus 40 (SV40) early promoter (Gelinas and Temin, 1988; Hannik and Temin, 1989; Rushlow et al., 1989). Murine c-rel has also been shown - by producing the c-rel protein in bacteria - to strongly activate transcription from kB sites whereas v-rel and other mutants of c-rel can suppress transcription activated by endogenous NF-kB (Inoue et al., 1991). V-rel and c-rel (translated in vitro) can form oligomers by interaction of their RH domains; a mutation in the N-terminal half of the protein results in a failure of oligomerisation. A regulatory domain has been identified in the RH domain of mouse c-rel, which can inhibit transactivation by rel proteins (Bull et al., 1990). Full-length c-rel protein, when linked to the DNA-binding domain of the yeast transcriptional activator GAL4, resulted in a weak transcriptional activation from a reporter gene, linked in cis to a GAL4 binding site. Deletion of the N-terminal half of the c-rel protein greatly increased the potency of activation by c-rel. This conserved, regulatory (inhibitory) domain could act as an additional control to allow transcription only in specific cell types, which perhaps contain the necessary cellular factors that may interact with the N-terminal domain, thereby exposing the C-terminal transactivation domain (Bull et al., 1990). The mechanism of suppression of kB

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sites and inhibition of transcription by v-rel may contribute to an understanding of the normal mechanism of transcriptional activation by c-rel, particularly since non-transforming v-rel mutants cannot inhibit kB-site directed transcription (Ballard et al., 1990).

The full-length open reading frame of the mRNA encoding p50 (the DNA binding 50kd subunit of NF-kB) is 105kd (Ghosh et al., 1990; Kieran et al., 1990). Removal of the C-terminal half of this precursor protein in vitro generates p50 and activates DNA binding (whereas c-rel does not require truncation of its C-terminal region to be able to bind to kB sites) and suggests a novel maturation processing of the 105kd protein in vivo. It is not known whether the carboxy terminal half of the precursor mRNA encodes a functional polypeptide, although this region does inhibit DNA binding and may encode I κ B. NF-kB binding activity constitutes a heteroligomer of p50 and p65 - it is not known if it is a heterodimer (p50-p65) or heterotetramer (p50)₂-(p65)₂ - formed by association of the RH domains. Both p50 and p65 alone are able to bind DNA, but they have distinct DNA binding properties. Gel electrophoretic mobility shift assays (GEMSA) were used to determine the DNA binding specificity of p50 and p65. Artificial palindromic kB motifs were created by duplicating the half sites (referred to as A and B) in the most frequent kB motif 5'-GGGACTTCC-3' (AB) into motifs with the sequence 5'-GGGACGTCCC-3' (AA) and 5'-GGAAATTTCC-

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3' (BB) (Urban and Baeuerle, 1990). GEMSA show that p50 homodimers have a higher affinity than p65 homodimers for DNA and bind most strongly to palindromic sequences of the 5' half-site (AA) of kB enhancer sites. In contrast, p65 homodimers bind with equal affinity to the low symmetry wt kB enhancer site (AB) and palindromic sequences of the 3' half-site (BB), and at least 50 fold less to the palindromic AA motif. However, p50-p65 heterodimers prefer the wt enhancer site (AB) (Urban et al., 1991). Since most physiological important kB motifs are not perfect palindromes, but show a distinct sequence conservation between their half-sites, they are predominantly targets for p50-p65 heterodimers. Indeed, it is not even known whether p50 (also known as KBF1) homodimers even have a physiological role in gene activation; in co-transfection assays using a reporter gene driven by a minimal promoter and a multimerised KBF1 binding site together with an expression vector containing full-length or truncated KBF1 cDNA, no transactivation was detected in mouse L cells or F9 cells, human HeLa cells or monkey COS cells (Logeat et al., 1991). It is possible that the p50 subunit only displays DNA binding activity and is devoid of an activation domain; its association with p65 would then be necessary for transcriptional activation. p50 homodimers may even act as a nuclear repressor of transcription by binding to kB sites, thereby preventing the active heterodimer from binding and activating transcription.

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p50 and p85 bind DNA as homodimers. An internally deleted construct of p50, Δ SP, cannot bind DNA. Δ SP can homodimerise with p50 or heterodimerise with p85 (human c-rel) or v-rel, forming non-binding oligomers. This suggests that there are different domains for DNA binding and dimerisation (Logeat et al., 1991). In addition to heterodimerising to p85 or p65, p50 can heterodimerise with p75. In contrast to p65 (which binds strongly as a heterodimer with p50, but weakly as a homodimer) both p85 and p75 bind strongly as homodimers as well as heterodimers with p50 (Ballard et al., 1990; Logeat et al., 1991).

Co-transfection of v-rel with kB-CAT constructs in T cells results in a poor stimulation of CAT activity. Treatment of T cells with HTLV-1 tax protein or PMA, which activate endogenous NF-kB, results in a large stimulation of CAT activity from these kB-CAT constructs. However, in the presence of v-rel, stimulation of CAT activity by PMA/tax induced activity of NF-kB is suppressed (Inoue et al., 1991; Ballard et al., 1990). V-rel could thus interfere with the normal function of NF-kB by transcriptionally inactive v-rel homodimers competing for kB binding sites or by interacting with NF-kB constituents to form inactive heterodimers.

Inhibition of transcription by the Δ SP construct and other mutants can be used to identify genes that are normally regulated by members of the rel family; as shown in the inhibition of expression of MHC Class 1 genes in two

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cell lines and HIV-1 expression in a CD4⁺ T cell line that were transfected with Δ SP. C-rel, like NF-kB may, therefore (as described in section 1.3.2), have a role in B and T cell activation; chicken and mouse c-rel occur predominately in bone marrow derived tissues (Moore and Bose, 1989; Grumont and Gerondakis, 1989).

1.3.4 Differential subcellular localisation of rel family proteins.

Dorsal and NF-kB both show differential localisation, where the movement of the protein from the cytoplasm to the nucleus is a controlled regulatory event. The nuclear localisation of dorsal is critical for dorsal function (as described in section 1.2.5) since dorsal establishes dorsoventral axis formation as a result of a nucleocytoplasmic gradient of dorsal protein. From the dorsal to the ventral side of the embryo, there is an increasing amount of dorsal protein found in the nucleus as opposed to the cytoplasm, whereas the total amount of dorsal protein is constant. Upon T cell activation in humans, p50 (a component of NF-kB) and p75 are translocated from the cytoplasm to the nucleus within a few minutes, whereas uptake of a cleavage product of p50 and p85 is delayed. Furthermore, when cytoplasmic extracts are treated with denaturing agents, such as DOC (which disrupts complex formation), allowing the detection of NF-kB in gel-retardation assays, p50 cleavage product and p85 require a

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harsher treatment than p50 and p75, indicating that p50 cleavage product/p85 may be held in the cytoplasm by a different mechanism to p50/p75 (Molitor et al, 1990). In uninduced cells, NF-kB (p50-p65) is maintained in an inactive state in the cytoplasm due to its association with a 37kd inhibitor protein, IκB (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990; Zabel and Baeurele, 1990). Interestingly, at the dorsal side of the Drosophila embryo where there is no dorsal activity, dorsal is also retained in an inactive form in the cytoplasm, and this retention requires the activity of cactus; it remains to be seen whether cactus and IκB are homologous.

Inactive p50-p65 can become active after dissociation from the cytoplasmic protein, IκB. The many different agents - including bacterial LPS, the cytokines TNF-α, TNF-β, interleukin-1 and T cell mitogens such as; PMA, lectins and calcium ionophores - that can stimulate the nuclear DNA binding activity of p50-p65, must therefore trigger a common mechanism of releasing IκB from p50-p65 in the cytoplasm. This induction can even occur in the absence of protein synthesis (Bohnlein et al., 1988).

Phosphorylation of IκB, in vitro, by protein kinase C blocks its ability to inhibit p50-p65 DNA binding (Ghosh and Baltimore, 1990). Protein kinase C acts as an intracellular receptor for phorbol esters and some other stimulating agents, by the same method as the activation of diacylglycerol (DAG) second messengers (endogenous, less

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potent phorbol esters; reviewed in Bell, 1988).

Activation of NF- κ B by TNF- α , unlike PMA, seems to be independent of protein kinase C (Meichle et al., 1990). Even though TNF induces a rapid and transient activation of protein kinase C, depletion of the enzyme by chronic PMA treatment does not affect NF- κ B activation by TNF. N-acetyl-L-cysteine (NAC) has recently been shown to greatly inhibit both the PMA- and TNF- α -induced activation of the HIV-1 LTR (Roederer et al., 1991); at the same time, NAC was shown to specifically block the activation of NF- κ B (Staal et al., 1990). NAC causes an increase in intracellular glutathione (GSH) levels, which in turn control the concentration of reactive oxygen intermediates (ROI) via the GSH peroxidase. Subsequently, it was observed that treatment of cells with hydrogen peroxide (H_2O_2) could activate NF- κ B (Schreck et al., 1991). This activation is a specific event since it occurs at low extracellular concentrations of H_2O_2 ; other DNA-binding proteins (such as AP-1) are unaffected by the treatment; and other kinds of cellular stress (such as heat shock or chemical treatment) do not induce the activity of NF- κ B under the conditions tested, that is, κ B elements in regulatory genes appear to serve specifically as response elements for oxidative stress. Since H_2O_2 on its own, cannot activate purified NF- κ B-I κ B complex or that contained in a cytosolic fraction, a metabolite of H_2O_2 (ROI such as O_2^- or OH) or an intracellular reaction induced by H_2O_2 may cause the

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release of I κ B. Oxidative damage or a controlled proteolytic degradation of I κ B would represent an irreversible mechanism of NF- κ B activation, which would correlate with the observation by Baeuerle *et al.*, (1989) that I κ B released *in vivo* during a PMA treatment of pre-B cells cannot be reused to inhibit the activated NF- κ B.

NF- κ B may, therefore, be activated by ROI produced in response to the activation of protein kinase C (PKC) rather than directly by the kinase since there is no solid evidence for an *in vivo* participation of the kinase in the activation of NF- κ B. PMA and TNF- α may activate NF- κ B by a common pathway diverging upstream of I κ B and involving oxygen radicals (see Fig. 6) since both these agents stimulate the production of superoxide anions and H₂O₂ in granulocytes, fibroblasts and other cell types.

Furthermore, the activation of NF- κ B by both these agents is inhibited by the radical scavenger NAC and other thiol compounds. Finally, PKC and TNF- α both cause a rapid decrease in GSH (which controls the level of ROI within cells) levels in Jurkat T cells (Staal *et al.*, 1990). The common mechanism of releasing I κ B from p50-p65 in the cytoplasm may, therefore, involve the use of ROI as second messengers; they fit the criteria for second messengers since they are small, diffusible and ubiquitous molecules that can be synthesised and destroyed rapidly (although high concentrations of ROI can become cytotoxic).

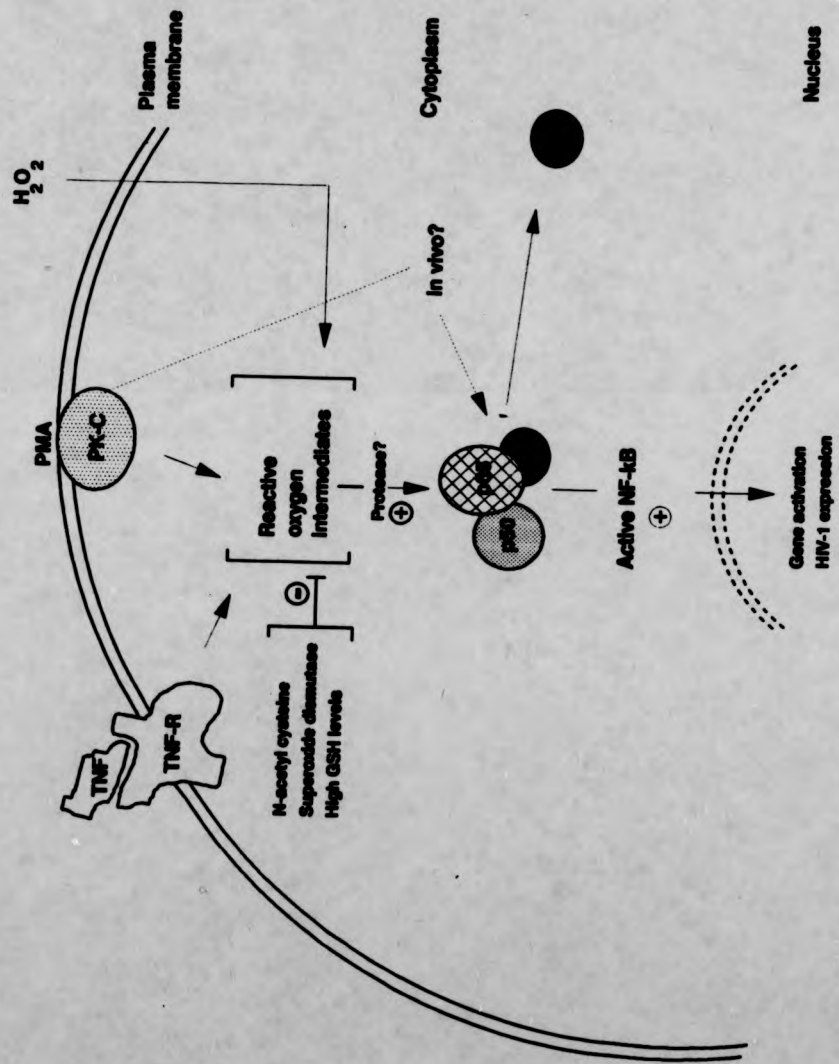
When there is excess p65-I κ B in a cell, phosphorylation

Figure 6.

Model proposed for involvement of reactive oxygen intermediates (ROI) as second messengers in the activation of NF- κ B.

**TNF - tumour necrosis factor
PMA - 12-phorbol-13-myristate acetate
PK-C - protein kinase C**

(Adapted from Schreck et al., 1991).



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of I κ B by protein kinase C may result in an increase of free p65, which could then activate p50-p65-I κ B that has not yet reached the membrane-bound kinase. In this way, p65 could act as a messenger between protein kinase C and p50-p65-I κ B. p65, rather than p50, interacts with I κ B since p50-p65 binding can be inhibited by I κ B, whereas (p50)₂ cannot. Furthermore, preincubation of I κ B with p65 prevents inactivation of p50-p65 and excess p65 causes p50-p65-I κ B to become active, presumably by associating with transiently released I κ B (Urban and Baeuerle, 1990).

This mechanism is an example of cell-type specific activation since in most cells there is an equimolar ratio of p65 to I κ B but in B cells this ratio is perturbed, resulting in higher levels of the active p50-p65 dimer. Nolan *et al.* (1991), have suggested that I κ B may inhibit DNA-binding activity of p50-p65 by impairing the DNA-binding site of p65, rather than acting in the cytoplasm, although no nuclear localisation has yet been observed for I κ B. It has been shown, however, that I κ B specifically binds to a site in the RH domain of p65 since I κ B can inhibit truncated p65; this site is not present in p50. The alternative view is that I κ B may passively mask a NLS or interact with a cytoplasmic structure such as microtubules, causing I κ B and proteins attached to it, to be retained in the cytoplasm. In this model, activation of p50-p65, is regulated by the inactivation of the cytoplasmic retention protein, I κ B.

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Recent cloning of a cDNA, MAD-3, that encodes I κ B-like activity has identified a domain containing five tandem repeats of the cdc10/ankyrin motif, which is 60% similar to an ankyrin repeat region in the C-terminal half of the 105kD precursor of p50 (Haskill et al., 1991). Ankyrins are known to constitute a family of proteins that regulate interactions between a variety of membrane structures (receptors) and the cytoskeleton (Lux et al., 1990). The developmentally important proteins, notch and lin-12, also contain ankyrin repeats. Ankyrin repeats of MAD-3 and the 105kD precursor of p50 could bridge the two proteins in close proximity in the cytoplasm and they may act as receptors involved in intracellular signalling. The activation of p50-p65 may, therefore, occur through a pathway that integrates receptor signalling and induction of specific genes through cytoskeletal-bound transcription factors. The in vitro translated product of MAD-3 shows identical properties to I κ B, including the specific inhibition of p50-p65 and it also contains a putative protein kinase C phosphorylation site in its C-terminal end. However, absolute proof that MAD-3 is identical to I κ B will require the complete purification (at present, I κ B has only been partially purified) and the cloning of its cDNA. It is possible that there are multiple I κ B activities which may be distributed in a cell-type specific manner. At present, two forms of I κ B are known : I κ B- α and I κ B- β (for review see Baeuerle, 1991). I κ B- β comprises only 20-

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30% of the total inhibiting activity in placenta cytosol. Phosphatase treatment inactivates the inhibitory activity of I κ B- β but not I κ B- α . p65 serves as a receptor for I κ B- α and I κ B- β ; I κ B- α specifically inhibits p50-p65 DNA binding activity, whereas I κ B- β can also inhibit the DNA binding of p75^{c-rel} (see below; Kerr *et al.*, 1991).

Features in v-rel and c-rel proteins that are involved in their cytoplasmic or nuclear localisation have been identified, but the conditions favouring nuclear localisation have yet to be defined. The protein product of v-rel, pp59^{v-rel}, is a phosphoprotein that is located in the cytoplasm of transformed avian spleen cells and in the nucleus of nontransformed, but infected chicken embryo fibroblasts (Gilmore and Temin, 1986). When overexpressed, chicken c-rel is located primarily in the cytoplasm of avian fibroblasts. However, subcellular localisation is irrelevant for transformation by Rev-T because both cytoplasmic and nuclear forms of v-rel can induce cellular transformation (Gilmore and Temin, 1988). As in the case of dorsal, C-terminal regions may be required to localise these proteins in the cytoplasm since truncated C-terminal c-rel and dorsal proteins are automatically translocated to the nucleus (Capobianco *et al.*, 1990). It is not known under what circumstances c-rel moves to the nucleus in fibroblasts. Inhibition of nuclear localisation of c-rel proteins may involve proteins interacting with the C terminus or the negative regulatory domain, which may

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interact with the C-terminus. This is surprising since one would have predicted that the conserved domain would be involved in the conserved property of nuclear localisation.

pp59^{v-rel} associates with other cellular proteins - including proteins of the sizes 115kD and 124kD and a 36-40kd protein, which is phosphorylated (pp40) - as detected by using v-rel antiserum in immunoprecipitations, which vary according to the subcellular localisation of v-rel (Morrison et al., 1989). C-rel is also found associated with these proteins in T and B lymphocytes, indicating that this association of proteins is not sufficient to generate a transformed phenotype. C-rel may not be associated with other cellular proteins in all tissues; the state of differentiation of a cell could influence the complex formation that results with c-rel. In cells of haemopoietic origin, pp59^{v-rel} and p75^{c-rel} may be regulated in a different manner by the association of cellular proteins and the replacement of pp59^{v-rel} for p75^{c-rel} could lead to a transformed phenotype.

In Rev-T transformed cells, the majority of pp59^{v-rel} is complexed with pp40 and a small fraction of p75^{c-rel} and a minority of pp59^{v-rel} is complexed with p124, p115 and a large fraction of p75^{c-rel}. About 10% of pp59^{v-rel} occurs in the nucleus and is complexed to pp40. p75^{c-rel} is not associated with pp40 in the nucleus, but like pp59^{v-rel} does occur in two distinct cytoplasmic complexes in normal haemopoietic cells. Unlike pp59^{v-rel}, the majority of

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p75^{c-rel} is complexed with p124 and p115, but about 40% of p75^{c-rel} is associated with the phosphorylated protein, pp40. The RH N-terminal region of c-rel is involved in binding to pp40 since in the presence of ionic detergents, which do not cause the dissociation of pp40 complexes, antiserum against only the N-terminal region of v-rel cannot precipitate native p75^{c-rel}. In contrast, in the presence of non-ionic detergents, which dissociate pp40 complexes, the N-terminal region becomes accessible to antiserum raised against the N-terminus of v-rel and p75^{c-rel} can be precipitated (Davis et al., 1990). The phosphorylated pp40 protein may be homologous to IκB-β, since it is also phosphorylated, of about the same size (37kD), and is also dissociated from a complex of proteins (p50-p65-IκB) in the presence of non-ionic detergents (eg. DOC). Furthermore, the binding of IκB-β to either p75^{c-rel} or p50-p65 dimer can be prevented by anti-pp40 antiserum (Kerr et al., 1991).

Chicken p75^{c-rel} and its homologue in other species may, therefore, act through association with specific cellular proteins and these proteins could define the function of c-rel and the mechanism of transformation by v-rel. It is not known whether regulation of c-rel activity is dependent on differential subcellular localisation in the same way as dorsal and p50-p65, but it may be present in the nucleus of specific cell types as a result of associations with different combinations of proteins.

Materials

CHAPTER 2: MATERIALS.

2.1 General materials.

Restriction enzymes were from Amersham International (U.K.), Northumbria biologicals limited (NBL) and Bethesda research laboratories, Maryland U.S.A. (BRL). E.coli DNA polymerase I and T4 DNA ligase were obtained from Amersham International, SP6, T3 and T7 RNA polymerases were from BRL, sequencing grade DNA polymerase Klenow fragment was from NBL and AMV reverse transcriptase was from Life sciences Inc. (U.S.A.).

All radioisotopes were supplied by Amersham International at the following specific activities;

α -³²P-dGTP and α -³²P-dCTP; 3000 Ci/mmol, γ -³²P-ATP; 5000 Ci/mmol, α -³⁵S-dATP; 1000 Ci/mmol, ³⁵S-methionine; >1000 Ci/mmol.

Nitrocellulose sheets (Hybond-C) and Nylon filters (Hybond-N) were obtained from Amersham.

Type II agarose (medium EEO) was supplied by Sigma chemical company and low melting point agarose was from FMC Bioproducts. Materials for bacteriological media were from Difco laboratories (Michigan, U.S.A.) and Oxoid limited (England).

E.coli transfer RNA (type XX) was supplied by Sigma.

X-ray film was from Fuji photo company limited (Japan).

Acrylamide was supplied by Fisons, and bisacrylamide by Kodak.

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All other chemicals and reagents were from BDH ("Analar" grade) or from Sigma chemical company unless otherwise stated.

2.2 Stock Solutions.

1x Barth X (BX) - 88mM NaCl, 1.0mM KCl, 2.5mM NaHCO₃, 15.0mM Tris.HCl pH 7.6, 0.3mM CaNO₃, 0.41mM CaCl₂, 0.82mM MgSO₄.

TE - 10mM Tris.HCl pH7.5, 1mM EDTA pH8.

NaE - 0.3M Na acetate, 10mM EDTA, pH 7.

10x TBE - 108g/l Tris base, 55g/l boric acid, 9.5g/l EDTA, pH8.

20x SSC - 3M NaCl, 0.3M Na Citrate, pH7.

50x Denhardtts - 1% each of Ficoll, polyvinylpyrrolidone, bovine serum albumin.

10x MOPS - 0.2M 3-(N-morpholino) propanesulphonic acid, 50mM sodium acetate, 10mM EDTA, pH7

Deionised formamide - Formamide was stirred with Amberlite monobed resin MB-3 or MB-4 (5g per 100 ml of formamide) until the pH was 7, filtered through Whatman No. 1 filter paper and then stored at -20°C.

Filtered formaldehyde - A 40% (w/v) solution of formaldehyde was filtered twice through Whatman No. 1 paper.

2.3 Bacteriological Media.

LB was prepared by dissolving 10g of NaCl, 10g of

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bactotryptone and 5g of yeast extract in 1 litre of distilled water. For plates this was supplemented with 15g/litre of bacto agar.

LB-Mg was prepared the same way as LB except that $MgSO_4$ was added to a final concentration of 10mM.

2x TY was prepared by dissolving 8g of NaCl, 16g of bactotryptone and 10g of yeast extract in 1 litre of distilled water.

H-Agar was made by adding 8g of NaCl, 10g of bactotryptone and 15g of bacto agar to 1 litre of distilled water

NZY broth was prepared by dissolving 5g of NaCl, 2g of $MgSO_4 \cdot 7H_2O$, 5g of yeast extract and 10g of NZ amine in 1 litre of water. For NZY plates this was supplemented with 15g per litre of bacto agar. NZY top agarose was made by adding 0.7g per 100 ml of agarose (type II) to NZY broth.

H-top agar was prepared by dissolving 10g of bactotryptone, 8g of NaCl and 6g of agar (or 6g of agarose for H-top agarose) in 1 litre of distilled water.

Teriffic broth (TB) was occasionally used to grow bacteria for large scale plasmid isolation. It was prepared by dissolving 12g of bactotryptone and 24g of yeast extract in 800 ml of distilled water, adding 3ml of glycerol and then autoclaving. Just before innoculating this with bacteria, 100ml of 0.17M KH_2PO_4 and 100ml of 0.72M K_2HPO_4 (both previously sterilized by autoclaving) were added.

All media were autoclaved before use.

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Antibiotics were used in plates and media at the following final concentrations. Ampicillin; 100ug/ml, tetracycline; 15ug/ml.

2.4 Bacterial, plasmid and phage genotypes.

2.4.1 E. coli strains.

JM101 - supE, thi, $\Delta(\text{lac-proAB})$, [F', traD36, pro AB, LACiqZDMI5] 17-18 ditto, not tra D36.

MC1061 - F⁻, ara D139, $\Delta(\text{ara, leu})7696$, $\Delta\text{lac Y74}$, gal U⁻, gal K⁻, hsr⁻, hsm⁺, strA.

BB4 - supF58, supE44, hsdR514 (rk⁻, mk⁻), galK2, galT22, trpR55, metB1, tonA, lambda⁻, D(arg-lac)U169 [F', proAB, lacIqZDMI5, Tn10(tet^r)].

TG2 - supE, $\Delta(\text{lac-proAB})$ hsd, $\Delta(\text{src-recA})$ 306::Tn10(tet^r) [F' traD36 proAB⁺ lacI^q lacZ M15 r_k⁻ r_m⁻ Rec⁻].

B121(DE3) - F⁻ hsdS gal, r_B⁻m_B⁻, lacUV5-T7 gene 1 cloned into int (see Studier and Moffat, 1986). -

2.4.2 Plasmid vectors.

pBR322 - General purpose cloning vector (Bolivar, 1978).

pBluescript SK⁻ - The plasmid generated by automatic excision from lambda ZAP. Has an extensive polylinker, the lacZ colour selection system and promoters for both T3 and T7 RNA polymerases (Stratagene, La Jolla, CA, U.S.A).

pET-3 series - Plasmids designed for the high level expression of cloned sequences in E. coli (Rosenberg et al., 1987). 3 vectors with a Bam HI site in each reading

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frame to make in frame fusions to the first 11 amino acids of gene 10 of phage T7. The fusion gene is transcribed by T7 RNA polymerase, and can therefore be expressed in the E. coli strain BL21(DE3). This strain contains the T7 RNA polymerase gene incorporated into the genome, and under the control of the lacUV5 promoter (inducible with IPTG).

pSP64T - This vector was designed to provide 5' and 3' flanking regions from a β -globin mRNA which is efficiently translated, to any cDNA which contains its own initiation codon and therefore yields high expression of the incorporated message by use of the SP6 promoter (Krieg and Melton, 1984).

2.4.3 Bacteriophage vectors.

M13mpl8 and M13mpl9 - bacteriophage M13 vectors designed for sequencing by the dideoxy chain termination method (Messing et al., 1981)

2.4.4 Plasmid and Bacteriophage Recombinants.

pTG7 - contains middle v-rel (Wilhelmsen et al., 1984). Gift of T. Gilmore and H. Temin.

X. laevis mature oocyte cDNA library in lambda ZAP (vector described in Short et al., 1988). Cloned sequences can be automatically excised from the phage vector and converted to the plasmid vector pBluescript, for ease of handling. Gift of J. Shuttleworth.

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CHAPTER 3; METHODS.

3.1 Oocytes, Eggs And Embryos.

3.1.1 Oocytes

Oocytes were obtained by anaesthetising a female Xenopus laevis with MS222 and surgically removing part of the ovary. The oocytes were manually stripped from the ovary in full-strength Barth X (BX), washed and kept at 14°C.

3.1.2 Eggs and embryos.

Female X. laevis were induced to ovulate by subcutaneous injection with 250 units of serum gonadotrophin (Intervet U.K. Ltd) 48 hours before laying followed by 250 units of chorionic gonadotrophin B (Intervet U.K. Ltd) 16 hours before laying. Eggs were laid in full-strength BX. For a 'natural mating', male X. laevis were injected with 250 units of chorionic gonadotrophin 8 hours before laying.

For in vitro fertilisation a male X. laevis was killed by injection with 0.5ml of 250mg/ml phenobarbitone (euthatal, May and Baker), and the testis removed and kept in full strength BX on ice. 50 to 100 eggs were transferred to a petri dish and dredged with pieces of teased testis for 30 seconds. After leaving for 1 minute, a small amount of distilled water was added to activate the sperm. Two minutes later the dish was flooded with distilled water.

Fertilised eggs were de-jellied in 2% (w/v) cysteine (pH adjusted to 8 with NaOH) and then washed at least 4 times

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in full strength 1/10x BX. Embryos were maintained into 1/10x BX to avoid exogastrulation.

Embryos were staged according to Nieuwkoop and Faber (1956).

3.2 Microinjection of Xenopus Oocytes.

Oocytes that had been obtained as described in section 3.1.1 were microinjected using a fine, drawn out glass capillary mounted on a micro manipulator. The needle was linked via a thin gauge pipe to a syringe driven by a vernier, and the system partially filled with oil. Oocytes were transferred to a piece of moist filter paper on a microscope slide and about 20nl of RNA (dissolved in DEPC-treated water) injected into the cytoplasm (vegetal half) under a binocular microscope.

3.3 Microinjection Of Fertilized Eggs.

Batches of eggs were fertilized in vitro and de-jellied soon after rotation. Embryos just beginning the first cleavage were transferred to full-strength BX supplemented with 5% (w/v) ficoll (m.w 40,000) and injected with about 20nl of RNA (in DEPC treated water). Embryos were bipolar injected at the 2 cell stage. Immediately after injection embryos were transferred to a 14°C incubator. They were kept in 5% ficoll at 14°C until they had reached stage 6 since this reduces leakage of cytoplasmic material from the hole made by microinjection, after which embryos were

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washed in 1/10x BX and incubated in 1/10 BX at 14°C for several days. Dead embryos were discarded regularly.

3.4 Isolation Of RNA.

3.4.1 Isolation of RNA from Xenopus oocytes, eggs and embryos.

Groups of eggs, embryos or oocytes were transferred to a glass homogeniser and homogenised in approximately 4 volumes of Kressman's buffer (10mM Tris.HCl pH7.5, 1.5mM MgCl₂, 10mM NaCl, 1mg/ml proteinase K (Boehringer)). After adding SDS to a final concentration of 2% (w/v) the homogenate was microfuged for 1 minute and then either incubated at 37°C for 40 minutes or extracted immediately with an equal volume of neutral phenol. One or two more phenol extractions were performed as required. The aqueous phase was extracted at least once with chloroform and then nucleic acids precipitated with 2 volumes of ethanol in the presence of 0.3M sodium acetate (pH6.5).

3.4.2 Selection Of Polyadenylated RNA By Oligo dT Cellulose Chromatography

This was carried out essentially as described by Maniatis et al. (1982). A 2ml oligo (dT) cellulose column was poured in a disposable plastic column and equilibrated with loading buffer (20mM Tris.HCl pH7.6, 0.5M NaCl, 1mM EDTA, 0.1% SDS in DEPC treated water). Columns were used several

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times and stored at 4°C. RNA (dissolved in DEPC treated water) and an equal volume of 2x loading buffer (40mM Tris.HCl pH7.6, 1M NaCl, 2mM EDTA, 0.2% SDS) were mixed and applied to the column, warming the column with a heating lamp as necessary. The column was washed with 5 to 10 column volumes of loading buffer and then with 5 column volumes of wash buffer (20mM Tris.HCl pH7.6, 0.1M NaCl, 1mM EDTA, 0.1% SDS). Flow-through was collected in 1ml aliquots. The A₂₆₀ of these fractions was measured as they were collected so that the amount of RNA coming off the column could be assessed. The column was washed until the A₂₆₀ was less than 0.1. Polyadenylated RNA annealed to the column was then eluted with 2 to 3 column volumes of elution buffer (10mM Tris.HCl pH7.6, 1mM EDTA pH8, 0.05% SDS). These eluted fractions were pooled and the RNA concentration determined by measuring the A₂₆₀. Finally, the RNA was split into aliquots, precipitated with ethanol and stored at -20°C until required.

3.5 Isolation Of Genomic DNA From Adult Xenopus Blood.

An adult female was anaesthetised by immersion in 0.2% (w/v) MS222 for 20 to 30 minutes. The thorax was then opened on the ventral side to expose the heart. A 10ml syringe, containing 3ml of 1mg/ml heparin in NMT (100mM NaCl, 10mM Tris.HCl pH7.4, 3mM MgCl₂), with needle attached, was inserted into the beating heart. Blood was gently collected into the syringe and transferred to cold

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universals. The cells were pelleted by centrifugation at 2000 rpm for 5 minutes, resuspended in 10ml of cold NMT and re-centrifuged. This was repeated until the supernatant was no longer pink. The cells were lysed by resuspending in 10ml of distilled water and leaving at room temperature for 5 minutes. 100ml of neutral phenol was transferred to a glass bottle and the lysed cell suspension added to this. After mixing vigorously the DNA was sheared by taking the emulsion into a syringe through a coarse grade needle twice. The emulsion was transferred to oakridge tubes and centrifuged at 8000 rpm for 20 minutes at room temperature. The aqueous phase was carefully transferred to a fresh tube and re-extracted with an equal volume of neutral phenol as before. The aqueous phase was transferred to a glass beaker and approximately two volumes of ethanol was added to precipitate the DNA. High molecular weight DNA was spooled onto the end of a pasteur pipette and transferred to a plastic universal. The DNA was dissolved in 10ml of TE (10mM Tris.HCl pH7.5, 1mM EDTA) by shaking gently at room temperature for several hours. Ribonuclease A was added to a final concentration of 5ug/ml and the solution incubated at 37°C for 30 minutes. An equal volume of 2x PK buffer (0.2M Tris.HCl pH7.5, 25mM EDTA, 0.3M NaCl, 2% (w/v) SDS) and 0.1 volumes of 2mg/ml proteinase K were added and the solution incubated at 37°C for 1 hour. The solution was then extracted with an equal volume of neutral phenol and the phases separated by centrifugation at 8000 rpm for 20

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minutes. The aqueous phase was transferred to corex tubes and the DNA precipitated with ethanol and recovered by centrifuging at 10 000 rpm for 20 minutes. Pellets were resuspended in 5ml of TE. The DNA concentration was determined by measuring the absorbance at 260 nanometers (assuming that a 50ug/ml solution has an A_{260} of 1) and the DNA solution stored at 4°C.

3.6 Gels used for resolving nucleic acids.

3.6.1 Formaldehyde Agarose RNA Gels.

RNA samples (up to 20ug) were resuspended in 10ul of DEPC treated water, to which 20ul of denaturing mix (66% deionised formamide pH7, 1.3x MOPS, 20% formaldehyde) and 3ul of loading mix (50% glycerol, 0.2% bromophenol blue) were added. The samples were loaded immediately on a 1.5% (w/v) agarose, 15% formaldehyde gel made in 1x MOPS buffer (1x MOPS is 20mM MOPS, 5mM sodium acetate, 1mM EDTA). The samples were electrophoresed at 40mA (100 volts) for 5 to 6 hours and the gel stained by first soaking in 250ml of 10% glycine for 10 minutes then adding 100ul of 10mg/ml ethidium bromide. After 10 minutes the gel was destained by washing twice, for 15 minutes each, in distilled water and then photographed on an ultraviolet light box.

3.6.2 Non-denaturing Agarose Gels.

DNA samples, to which 0.2 volumes of loading buffer (50%

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glycerol, 1xTBE, 0.1% bromophenol blue) had been added, were separated in 0.5 to 2% (w/v) agarose gels containing 0.5ug/ml ethidium bromide made in 1x TBE buffer. Gels were run in 1xTBE buffer containing 0.5ug/ml ethidium bromide and examined and photographed on an ultraviolet light box.

3.6.3 Low Melting Point Agarose Gels.

These gels were used to isolate DNA fragments generated by restriction enzyme digestion. These were subsequently radioactively labelled for use as probes, or used for sub-cloning purposes.

DNA samples to which 0.2 volumes of agarose gel loading buffer had been added, were loaded on to a 1% (w/v) low melting point agarose gel made in 1x TBE buffer and containing 0.5ug/ml ethidium bromide. Gels were run in 1x TBE buffer containing 0.5ug/ml ethidium bromide at a maximum of 40mA. The gel was examined under U.V. light and the required bands excised in as small a gel slice as possible. Exposure of the gel to U.V. light was kept to a minimum to avoid U.V. induced DNA damage. Gel slices were transferred to 1.5ml eppendorf tubes and weighed to calculate the volume of the slice (assuming 1g = 1ml). 4 volumes of NaE were added and the slices melted by heating at 65°C for 10 to 15 minutes. After cooling to 37°C an equal volume of neutral phenol was added, the solution whirlimixed vigorously and left on ice for 10 minutes. The tubes were then microfuged for 5 minutes and the aqueous

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phase transferred to a fresh tube. The volume of the aqueous phase was reduced to about 300ul by several extractions with butan-1-ol and the DNA precipitated at -20°C after adding 2 volumes of ethanol.

3.6.4 Denaturing polyacrylamide gels.

6 to 10% polyacrylamide (19:1 bis) gels containing 42% (w/v) urea in 1x TBE were poured, using a 25ml pipette, between 20x40cm gel plates with 0.4mm spacers. After heating for 3 minutes at 96°C , nucleic acid samples in denaturing gel loading buffer (90% deionised formamide, 10mM EDTA pH8, 0.01% xylene cyanol, 0.01% bromophenol blue) were loaded onto the gel and electrophoresed at 38 watts in 1x TBE. Gels were fixed in 10% glacial acetic acid (v/v), 10% ethanol (v/v) for 15 minutes, transferred to a sheet of 3MM Whatman paper, dried at 80°C on a vacuum drier and then exposed to X-ray film.

3.7 Northern Blotting.

After staining with ethidium bromide, formaldehyde-agarose gels (see section 3.6.1) were soaked in 250ml of 20x SSC for 20 minutes and the RNA transferred to nitrocellulose (Hybond-C, Amersham) as described by Thomas (1980). The gel was placed on a moistened filter paper wick, supported on a platform, with the ends of the wick immersed in 20x SSC. The edges of the gel were sealed with cling-film. A piece of nitrocellulose, cut to the same size

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as the gel, was lowered onto the gel, ensuring that no air bubbles formed. Two pieces of 3MM, the same size as the gel, were soaked in 2x SSC and layed on top of the nitrocellulose. Four pieces of dry 3MM were placed on top of this and then tissue paper was stacked on top of this to a depth of about 5cm and weighed down with a house brick on top a glass plate. The gel was capillary blotted overnight after which the nitrocellulose filter was briefly dried at room temperature and then baked at 80°C under vacuum for 2 hours.

Filters were prehybridised and hybridised in heat sealable plastic bags containing the appropriate solution and at the required temperature. Prehybridisation was in 20 to 50ml of 6x SSC, 5x Denhardtts, 50% deionised formamide (pH7), 100ug/ml E.coli tRNA and 0.1% SDS at 37°C for 4 hours or overnight. The prehybridisation solution was discarded and replaced with 5 to 10ml of the same solution to which radiolabelled nucleic acid probe had been added and the filters hybridised at 37°C overnight.

Filters were initially washed twice in 2x SSC, 0.1% SDS for 5 minutes each at room temperature and then twice for 30 minutes each in the final wash conditions (which depended upon the stringency required). After the final wash, filters were blotted on 3MM filter paper, and while still damp, wrapped in cling film and exposed to X-ray film with an intensifying screen at -70°C.

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3.8 Southern Blotting.

DNA samples separated in non-denaturing agarose gels as described in section 3.6.2 were transferred to nitrocellulose or nylon membranes using the method of Southern (1975). To increase the efficiency of transfer of high molecular weight DNA (especially with genomic Southern blots) the gel was first soaked in 500ml of 0.25M HCl for 15 minutes at room temperature on a shaker. This partially hydrolyses the DNA, generating smaller fragments which transfer more efficiently. After a brief wash with distilled water the gel was incubated in 500ml of denaturing solution (1.5M NaCl, 0.5M NaOH) with gentle shaking for 30 minutes. This was repeated with fresh denaturing solution, the gel rinsed with distilled water and then neutralised by gently shaking in 500ml of neutralising solution (3M NaCl, 0.5M Tris.HCl, 1mM EDTA, pH 7) for 45 minutes. This neutralising step was repeated with fresh neutralising solution and the gel was then capillary blotted using 20x SSC as the transfer buffer, exactly as described for northern blotting (see section 3.7).

DNA was fixed to nitrocellulose filters by baking at 80°C under vacuum for 2 hours. DNA was fixed to nylon membranes wrapped in cling film by exposure to ultraviolet light, DNA side down on a trans-illuminator for 3 minutes.

Prehybridisation, hybridisation, washing and autoradiography were carried out exactly as described for Northern blotting (see section 3.7)

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3.9 Large Scale Preparation Of Plasmid DNA And Purification By Caesium Chloride/Ethidium Bromide Centrifugation.

The method used was the alkaline lysis method as described by Maniatis et al. (1982). A single bacterial colony was inoculated into 10ml of sterile LB containing the appropriate antibiotic, and grown for several hours at 37°C. This 10ml culture was tipped into a 2 litre flask containing 500ml of LB or TB which was then shaken (200 rpm) overnight at 37°C. The next day, the cells were pelleted by centrifugation at 5000 rpm for 10 minutes. In the description which follows all volumes relate to a single 500ml bacterial culture. The pellets were resuspended in a total of 5ml of ice cold solution I (50mM glucose, 25mM Tris.HCl pH8, 10mM EDTA pH8, 5mg/ml lysozyme) and equal volumes transferred to two oakridge centrifuge tubes. After incubating at room temperature for five minutes 10ml of freshly prepared solution II (0.2M NaOH, 1% SDS) was added and the contents of the tubes mixed by inverting several times. The tubes were left on ice for 10 minutes after which 7.5ml of ice cold potassium acetate solution (made by mixing 30ml of 5M KAc, 5.75ml of glacial acetic acid and 14.25ml of distilled water) was added to each tube. The tubes were mixed by inverting several times and left on ice for 10 minutes. The pH of the solution was then tested with pH paper to ensure that the sodium hydroxide had been completely neutralized, adding more

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potassium acetate solution if necessary. Bacterial debris and chromosomal DNA was then pelleted by centrifuging at 13 000 rpm for 30 minutes. 18ml of supernatant were transferred to 30ml corex tubes and nucleic acids precipitated by adding 12ml of isopropanol. After 15 minutes at room temperature the tubes were centrifuged at 10 000 rpm for 30 minutes at 20°C. The pellets were resuspended in a total of 33ml of TE. Exactly 33g of caesium chloride was dissolved in this and 0.6ml of ethidium bromide (10mg/ml) added. Using a syringe, this solution was transferred to a 40ml Beckmann polypropylene heat sealable centrifuge tube. The tubes were then balanced to within 10mg and the tops heat sealed. They were then centrifuged at 45 000 rpm in a vertical rotor for 18 hours at 20°C.

The tubes were viewed under U.V. light. The lower band, which is supercoiled plasmid, was removed from the gradient using a syringe, and extracted at least three times with water saturated butan-1-ol to remove the ethidium bromide. The solution was then dialysed against 2 changes of TE for at least 5 hours at room temperature, transferred to corex tubes and the DNA precipitated with ethanol. The DNA was recovered by centrifuging at 10 000 rpm for 30 minutes at 4°C, dried briefly under vacuum and resuspended in 0.5ml of TE. The solution was then extracted with neutral phenol, and precipitated once more with ethanol. The DNA was pelleted by microfugation for 10 minutes, dried under

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vacuum and finally dissolved in 0.5ml of TE. The DNA concentration was determined by measuring the A_{260} , assuming that a 50ug/ml solution has an A_{260} equal to 1.0.

3.10 Primer Extension Analysis Of RNA.

The single stranded oligonucleotide to be used as the primer was end labelled with γ - ^{32}P -dATP (see section 3.32.3) and resuspended in TE. Hybridisation reactions were set up by mixing the RNA sample (about 5ug in a 5ul volume in DEPC treated water) and 100pg of the labelled primer in a total of 10ul of 1x hybridisation buffer (0.4M NaCl, 10mM PIPES pH6.4, 0.5mM EDTA pH8). The reactions were set up in eppendorf tubes and then transferred to drawn out glass capillaries, which were then heat sealed at both ends. The capillaries were placed in screw cap tubes full of water and incubated overnight at the hybridisation temperature, after a 10 minute incubation at 65°C. For 17mer and 18mer oligonucleotide primers, 45°C was found to be optimal.

After hybridisation the reactions were transferred to eppendorf tubes containing 80ul of reverse transcriptase buffer (50mM Tris.HCl pH8.3, 6mM MgCl_2 , 10mM DTT, 25ug/ml Actinomycin D, 2mM each of dATP, dCTP, dGTP and dTTP). 9 units of AMV reverse transcriptase was added and the reactions incubated at 42°C for 1 hour. Ribonuclease A was added to a final concentration of 2ug/ul and the tubes incubated for a further 10 minutes at 37°C after which DNA was precipitated at -20°C by adding sodium acetate pH6.5 to

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a final concentration of 0.3M and 2 volumes of ethanol. After a 15 minute microfugation the pellet was washed with 70% ethanol and re-microfuged for 5 minutes. The pellet was resuspended in 4ul of sequencing gel loading dye (90% deionised formamide, 10mM EDTA pH8, 0.01% xylene cyanol, 0.01% bromophenol blue), heated at 96°C for 3 minutes and then loaded onto a 6% denaturing polyacrylamide gel (see section 3.6.4). The gel was run at 38 watts until the bromophenol blue was about 2 inches from the bottom of the plate. The plates were prised apart and the gel incubated in fixer (10% v/v acetic acid, 10% v/v ethanol) for about 20 minutes. The gel was then dried on a vacuum drier at 80°C and exposed to X-ray film at -70°C, with an intensifying screen.

3.11 Primer extension/ Southern blot adaptation.

The equivalent of one embryo (whole or dissected) of total RNA was used in setting up the hybridisation reactions. The appropriate volume of RNA was added to 200ng of JHincI primer (see Appendix) in 1x hybridisation buffer (0.4M NaCl, 10mM PIPES pH6.4, 0.5mM EDTA pH8 in a total volume of 15ul. Hybridisations were carried out at 45°C for 1 hour. After hybridisation the reactions were transferred to eppendorf tubes containing 120ul of reverse transcriptase buffer (50mM Tris.HCl pH8.3, 6mM MgCl₂, 10mM DTT, 25ug/ml Actinomycin D, 2mM each of dATP, dCTP, dGTP and dTTP). 15 units of AMV reverse transcriptase was added

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and the reactions incubated at 37°C for 30 minutes. 1M NaOH was added to give a final concentration of 0.1M and the tubes incubated for a further 10 minutes at 37°C. The NaOH was then neutralised by the addition of HCl, after which DNA was precipitated at -20°C by adding sodium acetate (pH6.5) to a final concentration of 0.3M and 2 volumes of ethanol. After a 15 minute microfugation the pellet was washed with 70% ethanol and re-microfuged for 5 minutes. The pellet was resuspended in 20ul of water and Ribonuclease A was added to a final concentration of 2ug/ml. After a 5 minute incubation at 37°C, the samples were incubated at 65°C for another 5 minutes. The samples were then incubated on ice and 4ul of gel loading buffer was added. The samples were separated by electrophoresis on a 1.5% agarose gel, which was Southern blotted onto a nylon filter (see section 3.8). The filter was probed with radioactively labelled DNA and autoradiographed as described for Northern blotting (see section 3.7).

3.12 Subcloning Techniques.

3.12.1 Restriction Enzyme Digests.

These were carried out according to the manufacturer's instructions in a volume of 10-20ul. Plasmid DNAs were generally digested for 1 hour, whilst genomic DNA for Southern blotting was digested for 5 hours, adding a second aliquot of enzyme part way through the incubation.

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3.12.2 PCR Amplification.

PCR amplification of target DNA sequences was used to generate large amounts of insert required for ligation into the relevant vector. A 100ul PCR reaction was set up using 200ng of the relevant primers, 20-50ng of template, 1.25uM dNTPs and 5 units of Taq polymerase (Promega) in 1x ABHM buffer (10x ABHM buffer is 100mM Tris.HCl pH8.3, 500mM KCl, 40mM MgCl₂). 100ul of light paraffin was placed on top of the reactions to prevent evaporation. 30 cycles of the following programme were run: 94°C 1.5', 50°C 1' and 72°C 1.5'. At the end of the reaction, the aqueous layer was removed from beneath the paraffin with a Gilson and extracted with chloroform. The PCR products were recovered by precipitation with ethanol and redissolved in 20ul of TE.

3.12.3 Preparation of Plasmid Vectors for Subcloning.

Vectors that had been digested with two enzymes generating incompatible ends were run on a low melting point agarose gel and the linear vector fragment recovered. Vectors cut with a single restriction enzyme were treated with calf intestinal alkaline phosphatase (CIAP) by adding 1 or 2 units of enzyme into the digestion reaction at the end of the digestion period and incubating for a further 30 minutes at 37°C. Reactions were then increased in volume to 200ul, NaAc pH6.5 added to 0.3M, extracted with phenol,

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extracted with chloroform and the DNA precipitated with 2 volumes of ethanol.

3.12.4 Preparation of Target DNA for Subcloning.

Target DNA was generally a restriction fragment isolated from a low melting agarose gel (see section 3.6.3) or the product of a PCR reaction (see section 3.12.2). PCR products in TE were digested with the relevant restriction enzymes, phenol extracted and precipitated with ethanol.

For some purposes it was necessary to ligate target and vector with incompatible sticky ends. In this case 5' overhangs (on both target and vector) were 'blunted' by the addition of all 4 dNTPs (final concentration 1mM) and 10 units of Klenow fragment of DNA polymerase into the digestion reaction (at the end of the digestion period) and incubating for a further 30 minutes at room temperature.

3.12.5 Ligations.

10ul reactions were carried out in 1x C buffer (66mM Tris.HCl pH7.6, 6.6mM MgCl₂, 10mM DTT), containing 1mM ATP, vector DNA, target DNA and T4 DNA ligase. Usually, 20ng of vector and a concentration range of target DNA (1 to 10 fold molar excess over vector) was used. A control ligation that contained vector alone was always included. 1 unit was generally used and the reactions incubated at 14°C overnight.

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3.13 Transformation of E.coli with Plasmids.

3.13.1 $\text{CaCl}_2/\text{MgCl}_2$ Mediated Transformation.

This method was used for the E. coli strains MC1061, TG2 and BL21 (DE3). An appropriate volume of 2x TY or LB was inoculated with 0.01 volume of an overnight culture of the host bacteria and incubated at 37°C with vigorous shaking until the A_{550} reached 0.5. The culture was then cooled on ice for 10 minutes and the cells pelleted by centrifugation at 2000 rpm for 5 minutes. The pellet was resuspended in 0.5 volume of ice cold 100mM MgCl_2 and left on ice for 20 minutes. The cells were re-pelleted and resuspended in 0.1 volume of ice cold 100mM CaCl_2 . After incubating the cell suspension on ice for a further 20 minutes, 0.2ml aliquots were transferred to eppendorf tubes on ice. Half of the appropriate ligation reaction (5ul) or approximately 1ng of uncut plasmid DNA (as control) was added to an aliquot, followed by mixing and incubation on ice for 40 minutes. The cells were then heat shocked at 42°C for 2 minutes, transferred to ice for 5 minutes, and spread onto dried L-agar plates (containing the appropriate antibiotic) with an ethanol sterilized glass spreader. The plates were incubated inverted at 37°C overnight.

3.13.2 Hanahan High Efficiency Transformation.

This method is not appropriate for all strains (eg. MC1061) and was generally used for the high efficiency transformation of JM101 with plasmid and M13 vectors. The

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method is essentially as described by Hanahan (1983). A culture of host cells was grown as described in section 3.13.1, to A_{550} between 0.3 and 0.4, at which point the culture was cooled on ice for 15 minutes. Cells were pelleted by centrifugation at 2000 rpm for 5 minutes and resuspended in 0.3 of the original culture volume of transformation buffer (TFB; 10mM KMES pH6.3, 100mM KCl, 45mM $MnCl_4 \cdot 4H_2O$, 10mM $CaCl_2$, 3mM $HACoCl_3$). The suspension was left on ice for 15 minutes, cells were pelleted and resuspended in 0.08 original culture volume of TFB, transferred to a polypropylene tube and left on ice for 10 minutes. Dimethylformamide (DMF) was added to a final concentration of 4.75% (v/v) and the mixture left on ice for 10 minutes. A solution of 2.25M DTT in 10mM potassium acetate was added to 4.1% (v/v), the suspension mixed well and left on ice for a further 10 minutes. A second aliquot of DMF was then added to give a final concentration of 9.5% (v/v). The mixture was left on ice for 20 minutes and then 0.2ml aliquots transferred to eppendorf tubes on ice. Half of the appropriate ligation reaction (5ul) or approximately 1ng of uncut plasmid DNA (as control) was added to an aliquot, and the mixture incubated on ice for 40 minutes. The cells were then heat shocked at 42°C for 2 minutes and transferred to ice for 5 minutes. The transformed cells were then spread onto dried L-agar plates (containing the appropriate antibiotic) with an ethanol sterilized glass spreader, or in the case of M13 transformations plated in

Methods

top agarose (see section 3.14). The plates were incubated inverted at 37°C overnight.

3.14 Plating E.coli Transformed or Infected with Bacteriophage M13.

Each transformation or infection was added to 3ml of molten H-top agar at 45°C, to which had been added 30ul of X-gal (20mg/ml in DMF), 30ul of IPTG (24mg/ml in water) and 200ul of exponential host cells (in that order). The mixture was poured onto a dried H-agar plate, the plate was rocked gently to ensure an even covering of top agar and then allowed to set at room temperature for 10 minutes. Plates were incubated inverted at 37°C overnight.

3.15 Small Scale Isolation of Plasmid DNA and M13 RF DNA.

For the preparation of plasmid DNA, 2ml of 2x TY (containing the appropriate antibiotic) was inoculated with a single bacterial colony and grown in a shaking incubator at 37°C for 8 to 14 hours. Alternatively, for preparing M13 RF DNA, 2ml of 2x TY was inoculated with 20ul of an overnight culture of the host cells and phage (10ul of stock or a sterile toothpick which had been stabbed into a single plaque) and grown at 37°C with vigorous shaking (300 rpm) for 6 to 8 hours. 1.5ml of culture was transferred to an eppendorf tube and then microfuged for 5 minutes. In the case of M13 infected cells an aliquot of the supernatant was removed and stored at -20°C. This phage stock was also

Methods

used for the preparation of single stranded M13 DNA (see section 3.16). The pellets were resuspended in 0.2ml of STET (8% sucrose (w/v), 0.5% Triton X-100 (v/v), 50mM EDTA pH8, 10mM Tris.HCl pH8) and 10ul of fresh 10mg/ml lysozyme added, incubated on ice for 10 minutes, and then heated at 100°C for 40 seconds. Bacterial debris and chromosomal DNA were pelleted by microfugation for 10 minutes, this viscous pellet was removed using a toothpick and nucleic acids in the supernatant precipitated by adding 0.1 volumes of 3M NaAc (pH6.5) and 1 volume of isopropanol. The tubes were left at -20°C for at least 30 minutes and then microfuged for 10 minutes to recover the nucleic acids. The pellet was resuspended in 0.2ml of NaE (0.3M sodium acetate pH6.5, 1mM EDTA) and the solution extracted with an equal volume of neutral phenol, then an equal volume of chloroform. The nucleic acids were precipitated by adding 2 volumes of ethanol. After at least 30 minutes at -20°C, nucleic acids were recovered by microfugation for 10 minutes and the pellet resuspended in 40ul of TE. If necessary, RNA was removed by treatment with RNase A at a final concentration of 20ug/ml for 15 minutes at 37°C, and the solution re-extracted and precipitated.

3.16 Preparation of Single Stranded M13 Template DNA.

2ml of 2x TY was inoculated with 20ul of an overnight culture of the host strain (usually JM101) and phage particles (a toothpick inserted into a single plaque or

Methods

10ul of a frozen phage stock). Cultures were then incubated at 37°C with vigorous shaking (300 rpm) for 6 to 8 hours. The bacteria were pelleted by microfugation for 5 minutes and 1ml of the supernatant transferred to an eppendorf tube. Bacteriophage particles were then precipitated by the addition of 200ul of 20% (w/v) polyethylene glycol in 2.5M NaCl and incubation at room temperature for 20 minutes. Phage were pelleted by microfugation for 10 minutes. Supernatant was removed using a Gilson pipette, followed by a drawn out glass capillary after a 10 second recentrifugation. Pellets were resuspended in 0.2ml of NaE (0.3M sodium acetate pH6.5, 1mM EDTA), the suspension extracted with an equal volume of neutral phenol, followed by an equal volume of chloroform and the DNA precipitated by adding 2 volumes of ethanol. After at least 30 minutes at -20°C the DNA was recovered by microfugation for 10 minutes and dissolved in 10ul of TE. Template DNA was checked (for concentration and contamination with RNA) by electrophoresis on a 1% non-denaturing agarose gel prior to sequencing.

3.17 Complementation Tests on Single Stranded M13 DNA

M13 subclones with an insert in both orientations were selected using this method.

10ul of phage supernatant (in 2x TY) from each of two clones was mixed with 2ul of 1% SDS and hybridised at 65°C for 1 hour. 4ul of loading buffer was added and samples

Methods

electrophoresed on a 1% non-denaturing agarose gel. The presence of a slower migrating band (not present in either phage supernatant alone) indicated that the two clones had annealed, and therefore contained the insert in opposite orientation.

3.18 Plating Bacteriophage Lambda.

An appropriate volume of LB-Mg was inoculated with host cells (BB4) from an overnight culture and grown on a shaker at 37°C until the A_{550} reached 0.5. The cells were pelleted by centrifugation at 2000 rpm for 5 minutes and resuspended in 0.05 original culture volume of 10mM $MgSO_4$.

Bacteriophage particles (from phage stock in SM buffer; 50mM Tris.HCl pH7.5, 0.58% (w/v) NaCl, 0.2% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) gelatin) were mixed with these host cells and allowed to adsorb for 15 minutes at 37°C. Up to 100 000 phage were absorbed with 0.6ml of host cells. 0.6ml of infected cells were added to 6mls of molten top agarose equilibrated to 45°C, and this was then poured onto a dried 10cm x 10cm NZY plates. The plates were allowed to set and then incubated, inverted at 37°C until plaques of a suitable size formed.

3.19 Bacteriophage Lambda Plaque Lifts.

This procedure, used to transfer bacteriophage DNA from plates to nitrocellulose filters, is essentially as described by Benton and Davis (1975). The host strain BB4

Methods

was used. Bacteriophage lambda were plated (see section 3.18) using top agarose (not agar), and then incubated at 37°C for 5 to 6 hours until suitably sized plaques formed. The plates were then transferred to 4°C for at least 2 hours. Nitrocellulose filters were lowered onto the surface of the top agarose ensuring that no air bubbles formed. These were left in place for 1 minute during which time the position of the filter was marked by making three asymmetrical holes through the filter and into the agar with a syringe needle. The filters were removed and placed, DNA side up, onto a tray containing several sheets of filter paper (Whatman 3MM) moistened with denaturing solution (0.5M NaOH, 1.5M NaCl). The filters were left in place for 1 minute during which time a second nitrocellulose filter was applied to each plate. This replica filter was left in place for 2 minutes and orientated by making holes in the identical positions used for the first filter. The replica filter was denatured in the same way as the first filter. After denaturation the filters were transferred to a tray containing several sheets of 3MM moistened with neutralising solution (3M NaCl, 0.5M Tris.HCl pH7, 1mM EDTA), left in place for 4 minutes and then transferred to a second tray of neutralising solution for a further 5 minutes. Finally filters were transferred onto a tray of 3MM soaked in 2x SSC for 10 minutes. The filters were then allowed to air dry, and then baked at 80°C under vacuum for 2 hours.

Methods

Prehybridisation, hybridisation and autoradiography of filters were as described for Northern blotting (see section 3.7)

The region of a plate containing a positive plaque was identified by the orientation marks made in the filter and plate, an agar plug cut from the plate and transferred to 1ml of SM buffer containing 3ul of chloroform. This phage stock (stored at 4°C) was replated (at a lower density) and plaque lifts performed. Positive plaques were identified and the process repeated until the agar plug taken contained a single positive plaque.

3.20 Automatic Excision of Phagemids from Lambda ZAP

Clones.

This was carried out as described by the manufacturer. 0.2ml of BB4 culture (A_{550} = 0.8-1.0) was mixed with 100ul of lambda ZAP phage stock (from a single positive plaque, see section 3.19) and 10ul of R408 helper phage (7×10^{10} pfu/ml, supplied by Stratagene). The cultures were incubated in a 37°C shaker at 200 rpm for 4 to 6 hours, heated at 70°C for 20 minutes (to kill bacteria) and then centrifuged at 2,000 rpm for 5 minutes. The supernatant, which contains phagemid particles, was transferred to a fresh tube and stored at 4°C.

To convert phagemid to colony form, 0.2ml of a fresh BB4 culture (A_{550} about 0.5) was mixed with 0.1 to 100ul of the phagemid stock and the mixture incubated at 37°C for 15

Methods

minutes to allow adsorption of the phage. 10 and 100ul aliquots were spread onto dried L agar plates containing ampicillin (100ug/ml). The plates were incubated inverted at 37°C overnight.

3.21 Construction of an Exonuclease III Nested Deletion Series.

This method was first described by Linxweiler and Horz (1982) and in this case uses a double-stranded Nested Deletion Kit (Pharmacia). 3ug of circular DNA was linearised with two appropriate restriction enzymes, one of which produces a 3' overhang to protect this end from exonuclease III deletion. The digests were heated at 70°C for 10 minutes in order to inactivate the enzymes. An S1 nuclease/buffer mix was prepared in an eppendorf tube containing 33ul of S1 buffer (30mM KAc pH 4.6, 0.25M NaCl, 1mM ZnSO₄ and 5% glycerol), 66ul of distilled water and 5 units of S1 nuclease. 3ul aliquots of this mix were pipetted into 10 eppendorf tubes on ice. 24ul of 2x exonuclease III buffer, containing 8ul of exonuclease buffer (66mM Tris.HCl pH8.0, 0.66mM MgCl₂), 8ul of distilled water and 8ul of 0.3M NaCl was prepared. 20ul of this buffer was mixed with 20ul (2ug) of double-digested DNA and the mix equilibrated at room temperature for 3 minutes. A 2ul aliquot was removed as a 'time=0' sample and mixed with 3ul of S1 nuclease/buffer in a tube on ice. 200 units of exonuclease III were added to the remainder and

Methods

the incubation continued at 35°C. A 2ul sample was taken every 2 minutes and immediately mixed with 3ul of S1 nuclease/buffer on ice. All time point samples were kept on ice until the last sample was taken after 20 minutes. All samples were then incubated at room temperature for 30 minutes. 1ul of S1 stop solution (0.5M Tris.HCl pH8.0 and 125mM EDTA) was added to each of the samples which were then incubated at 65°C for 10 minutes.

Half of each timed sample was analysed on a non-denaturing agarose gel and the other half used for recircularisation by ligation.

3.22 DNA Sequencing by the Dideoxy Chain Termination Method

3.22.1 Sequencing Single Stranded M13 Templates.

This method was first described by Sanger et al. (1977). A set of deoxynucleotide and dideoxynucleotide mixes were assembled as shown below:

(A)	A°	C°	G°	T°
0.5mM dCTP	20ul	1ul	20ul	20ul
0.5mM dGTP	20ul	20ul	1ul	20ul
0.5mM dTTP	20ul	20ul	20ul	1ul
TE buffer	20ul	20ul	20ul	20ul

(B) Dideoxy NTP working solutions: 0.03mM ddATP, 0.1mM ddCTP, 0.075mM ddGTP, 0.5mM ddTTP. These concentrations were altered when necessary to suit a particular template.

Methods

(C) Chase mix: 0.5mM of all 4 dNTPs.

Annealing reactions (in an eppendorf tube) contained 1ul of single stranded template DNA, 6.5ul of sterile water, 1.5ul of freshly prepared Klenow reaction buffer (9ul of 10x core buffer (100mM Tris.HCl pH8, 50mM MgCl₂) and 1ul of 700mM B-mercaptoethanol), and 1ul of M13 17mer sequencing primer (2ng/ul). Annealing was carried out by placing tubes in a large shallow tray containing water at 80 to 85°C and leaving this to cool for about 30 minutes. 1ul of the A^o nucleotide mix and 1ul of the ddATP nucleotide solution was placed into a 0.5ml eppendorf tube (the "A" tube). This was repeated for the "C", "G", and "T" tubes using the appropriate nucleotide mixes. The annealed template/primer mix was centrifuged briefly to bring down any condensation, and then 1ul of α -³⁵S-dATP and 1ul of DNA polymerase Klenow fragment (5 units/ul) added. 2.5ul of this mix was added to each of the tubes containing the nucleotide mixes and the reaction allowed to proceed at 30°C for 20 minutes after which 1ul of chase mix was added and the tubes incubated for a further 10 minutes at 30°C. The reaction was terminated by adding 5ul of denaturing gel loading buffer (see section 3.6.4).

One third of each reaction was analysed on a 6% denaturing polyacrylamide gel made with a sharks tooth comb. The gel was generally run until the bromophenol blue in the loading buffer was at the bottom, and then a second

Methods

1/3 of the reaction loaded. Electrophoresis was continued until the bromophenol blue from this loading was at the bottom of the gel.

3.22.2 Extended M13 dideoxy sequencing.

This is an adaptation of the standard M13 dideoxy sequencing protocol which enables sequence to be read at greater distances from the primer. This method was obtained from the BRL M13 cloning/dideoxy sequencing manual. With the gel system available (40cm) it was only possible to read a maximum of 650 bases from the primer. The following termination mixes were prepared;

	A	C	G	T
dATP	25uM	250uM	250uM	250uM
dTTP	250uM	250uM	250uM	25uM
dCTP	250uM	25uM	250uM	250uM
dGTP	250uM	250uM	25uM	250uM
ddATP	300uM	-	-	-
ddTTP	-	-	-	500uM
ddCTP	-	100uM	-	-
ddGTP	-	-	150uM	-

The template and the primer were annealed by mixing 1ul of template DNA, 2ul of 5x sequencing buffer (200mM Tris.HCl pH7.5, 50mM MgCl₂, 250mM NaCl) and 4ng of primer in a final volume of 10ul. The tube was then placed in a

Methods

shallow tray of water at 80°C and left to cool at room temperature for about 40 minutes. For each template, four tubes were labelled A, C, G and T, and 2.5ul of the appropriate termination mix was placed in the bottom of each of these tubes. To the annealed primer/template mix was added 2ul of extension mix (1.5uM dCTP, 1.5uM dGTP, 1.5uM dTTP), 1ul of α -35S-dATP, 1ul of 0.1M DTT, 1ul of water and 2 units of DNA polymerase I Klenow fragment. The contents of the tube were mixed and the tube incubated at room temperature for 5 minutes. 3.5ul aliquots of this labelling/extension reaction were pipetted onto the sides of each of the tubes containing the termination mixes. The tubes were centrifuged briefly to mix the contents and then incubated at 37°C for 5 minutes. The reactions were then terminated by addition of 4ul of sequencing gel loading dye, heated at 96°C for 3 minutes and analysed on a 6% denaturing polyacrylamide gel using two loadings. These gels contained only 0.5x TBE and were run with 0.5x TBE in the top buffer tank and 1x TBE in the lower buffer tank. The first set of samples were run at 24mA for 1.5 hours after which the second set of duplicate samples were loaded on the same gel. The gel was then run at 18mA for a further 4.5 to 5 hours. The gel was then fixed in 10% glacial acetic acid (v/v), 10% (v/v) ethanol for 15 minutes, dried down and autoradiographed.

Methods

3.22.3 Sequencing plasmid DNA.

Plasmid DNA from a small scale isolation, treated with RNaseA, was used as template. Approximately 2ug of DNA in 200ul of NAE was extracted with neutral phenol and precipitated with 2 volumes of ethanol at -20°C . DNA was recovered by microfugation for 10 minutes and denatured by suspension in 20ul of 0.2M sodium hydroxide; 0.2mM EDTA. The solution was left at room temperature for 5 minutes, and then DNA precipitated by adding 2ul of 2M ammonium acetate (pH4.5) and 100ul of ethanol and leaving at -70°C for 30 minutes. DNA was recovered by microfugation for 15 minutes, and then the pellet was washed in 80% (v/v) ethanol and briefly dried under vacuum. DNA was suspended in 10ul of annealing mix (1.5ul of reaction buffer (see section 3.22.1), 6.5 ul of water and 2ul of M13 17mer sequencing primer (2ng/ul)). Primer was annealed to template by incubation at 37°C for 15 minutes, and then sequencing reactions carried out as described for single stranded M13 templates (see section 3.22.1).

3.23 Preparation of Synthetic RNA

Subclones in the vector pBluescript SK- (Stratagene) or pSP64T were linearised at an appropriate polylinker restriction site and transcribed in a reaction containing 1x transcription buffer (40mM Tris.HCl pH8, 6mM MgCl_2 , 20mM DTT), 0.5mM ATP, 0.5mM UTP, 0.5mM CTP, 0.05mM GTP, 62.5ug/ml BSA, 10 units of human placental RNase inhibitor

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(Pharmacia), 200 to 500ng template (linearised DNA), 0.5mM RNA Cap structure analogue (m7G(5')ppp(5')G, from New England Biolabs) and 10 units of SP6, T7 or T3 RNA polymerase (New England Biolabs). The reaction was incubated at 37°C for 1 hour, then GTP (to a final concentration of 0.5mM) and 10 units of SP6, T7 or T3 RNA polymerase added. The reaction was incubated for a further 30 minutes at 37°C. 25 units of DNase I was added, and the reaction continued at 37°C for 10 minutes. The reaction was stopped by the addition of 200ul of NaE (0.3M sodium acetate pH6.5, 1mM EDTA), extracted with an equal volume of neutral phenol, the aqueous phase extracted with chloroform, and RNA precipitated from the aqueous phase by the addition of 2 volumes of ethanol. After at least 30 minutes at -20°C, nucleic acids were recovered by microfugation for 10 minutes and resuspended in DEPC-treated water. RNA concentration was estimated by electrophoresising a fraction of the product of the transcription reaction on a non-denaturing agarose gel alongside standards and viewing the gel under U.V. light. Stock solutions were adjusted to 0.5mg/ml and stored at -70°C. Capped synthetic message prepared in this way was micro-injected into the cytoplasm of oocytes and into embryos at the 2-cell stage. Control uncapped transcripts were prepared in the same way, except that the Cap analogue was omitted.

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3.24 SDS-Protein Polyacrylamide Gel Electrophoresis.

3.24.1 Running Protein Gels.

A 20cm x 20cm gel was poured in two parts. Firstly the 16cm separating gel, which consisted of 10 to 15% acrylamide (37.5:1 bis), 0.3M Tris.HCl pH8.8, 0.1% (w/v) SDS), was poured. Before setting, the gel was overlaid with water-saturated butanol. After setting this was poured off and the separating gel was overlaid with the 4cm stacking gel (into which the slot former was inserted), which consisted of 3% acrylamide (20:1 bis), 0.125M Tris.HCl pH6.8, 0.1% (w/v) SDS. Protein samples were mixed with an equal volume of 2x loading buffer (0.125M Tris.HCl pH6.8, 20% glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.002% bromophenol blue), heated at 96°C for 3 minutes, cooled and loaded onto the gel, which was run at 10 to 40mA in 1x running buffer (50mM Tris, 400mM glycine, 0.1% (w/v) SDS).

3.24.2 Staining Protein Gels with Coomassie Blue.

Gels were incubated in stain (10% (v/v) acetic acid, 45% (v/v) methanol, 0.1% (w/v) Coomassie blue) for at least 1 hour on a slow shaker. The gel was then incubated in several changes of destain (10% (v/v) acetic acid, 45% (v/v) methanol) on a slow shaker, until background staining was adequately reduced. Gels were photographed wet, and then dried on a vacuum drier at 60°C.

Methods

3.24.3 Fluorography of Protein Gels.

Gels used to resolve proteins labelled with ^{35}S -methionine were fluorographed to enhance the signal seen on X-ray film.

Gels were stained with Coomassie blue or simply incubated in fixer (10% (v/v) acetic acid, 45% (v/v) methanol) for at least 1 hour and then transferred to DMSO for 30 minutes followed by another 30 incubation in fresh DMSO and then a 3 hour incubation in 22% PPO in DMSO. The gel was then incubated in water (to precipitate the scintillant) for 30 to 45 minutes, dried on a submersible vacuum drier at 60°C , and exposed to X-ray film at -70°C .

3.25 In Vitro Translation.

Synthetic RNA was translated in rabbit reticulocyte lysate by mixing 8ul of the lysate (Amersham) with 1ul of synthetic RNA (see section 3.23) and 1ul of ^{35}S -methionine, and incubating at 30°C for 45 minutes. After adding an equal volume of 2x SDS loading buffer and heating at 96°C for 3 minutes the sample was separated on an SDS polyacrylamide protein gel and visualised by fluorography (see section 3.24)

3.26 Western Blots.

Proteins resolved on SDS polyacrylamide gels (see section 3.24.1) were electrophoretically transferred to nitrocellulose. The gel was soaked in blotting buffer (20mM

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Tris, 150mM glycine, 20% (v/v) methanol) for 30 minutes. A sandwich of 3 sheets of filter paper (Whatman 3MM) followed by a sheet of nitrocellulose, the gel and 3 sheets of 3MM was assembled. All components were pre-wetted in blotting buffer. The sandwich was submerged in blotting buffer, in an electro-blotting tank, with the nitrocellulose between the gel and the positive electrode. The gel was blotted at 30 volts overnight (or 60 volts for 2 hours). Blots were stored wrapped in cling film at -20°C .

3.27 Immuno-detection of Proteins on Western Blots.

Non-specific protein binding sites of a Western blot (see section 3.26) were blocked by incubating in 100ml of TBS (20mM Tris.HCl pH7.6, 137mM sodium chloride), containing 0.1% tween-20 and 5% dried non-fat milk, on a slow shaker for 1 hour. The filter was washed, on a slow shaker, in 3 changes of 100ml of TBS containing 0.1% tween-20 (v/v), for 5 minutes each. The filter was then incubated (sealed in a plastic bag) with 10ml of TBS containing antiserum or affinity purified antiserum (usually 100ul) and incubated on a slow shaker for 1 to 2 hours. The filter was then washed, as before, in TBS containing 0.1% tween-20.

Bound antibodies were detected using an Amersham Blotting Detection Kit (for rabbit antibodies) according to the manufacturer's instructions. The filter was incubated with 10ml of TBS containing biotinylated anti rabbit immunoglobulin on a slow shaker for 30 minutes, and then

Methods

washed as before. The filter was incubated with 10ml of TBS containing streptavidin-alkaline phosphatase conjugate on a slow shaker for 20 minutes, and then washed as before.

Finally, the filter was incubated with 10ml of diethanolamine buffer (100mM diethanolamine.HCl pH 9.5, 5mM MgCl₂) containing nitro-blue tetrazolium (NBT) and 4-chloro-3-indoyl phosphate (BCIP), the reaction allowed to proceed as long as necessary, before stopping by washing in water.

3.28 Generation and Purification of a Polyclonal Antiserum.

3.28.1 Purification of a Fusion Protein Produced in E. coli for Use as an Antigen.

A fusion protein produced from a cloned fragment of X. laevis x-rel.2 in the pET-3b vector (Rosenberg et al., 1987) was purified for use as an antigen by preparative polyacrylamide gel electrophoresis.

E. coli BL21 (DE3) transformed with the fusion protein construct was grown by shaking at 37°C in 2x TY (containing 200ug/ml ampicillin) to A₆₀₀=0.8. IPTG was added to 1mM to induce T7 RNA polymerase (which transcribes message for the fusion protein) and growth continued for a further 3 hours. The cells were pelleted, resuspended in 0.01 volume of 1x SDS gel loading buffer, sonicated, heated at 96°C for 3 minutes and the proteins (from approximately 50ml original culture volume) resolved on a double thickness SDS polyacrylamide gel (see section 3.24.1). The T7 gene 10-x-

Methods

rel.2 fusion protein was visualised by briefly staining the gel with 0.05% Coomassie blue in water. The gel slice containing the fusion protein was excised.

Further purification was carried out by electroeluting the protein antigen from the polyacrylamide gel slice; a procedure adapted from Harlow and Lane, 1988. The excised gel slice was weighed and placed in a dialysis tubing bag containing 1ml of elution buffer (0.2M Tris-acetate pH 7.4, 1.0% SDS and 100mM DTT) per 0.1g of gel slice. The dialysis tubing was then placed cross-wise in a horizontal electrophoresis chamber. The contents of the tubing were electroeluted at 100mA in 1x electroelution running buffer (50mM Tris-acetate pH 7.4, 0.1% SDS and 0.5mM sodium thioglycollate) for 3 hours. At the end of this period, the gel slice was removed and stained with Coomassie blue to ensure that all the protein antigen had been eluted out of the acrylamide slice. The tubing was reclosed and the solution contained within it was dialysed against several changes of dialysis buffer (50mM ammonium carbonate, 0.02% SDS) overnight. The protein solution was removed from the tubing and lyophilised. The protein antigen was resuspended in 10ml of PBS and re-lyophilised. The purified protein pellet was finally resuspended in PBS to give a final concentration of 0.5mg/ml.

3.28.2 Immunisation of Rabbit.

For the first injection the purified solution of antigen

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was emulsified with an equal volume of Freund's complete adjuvant (see Harlow and Lane, 1988). For subsequent injections, slices were emulsified with Freund's incomplete adjuvant. Approximately 10ml of blood (for pre-immune serum) was taken from the ear of a New Zealand White rabbit before the first injection. The rabbit was given 3 injections of 50-100ug of protein at 10 day to 2 week intervals. Bleeding the rabbit took place 2 weeks after the third injection. Blood was allowed to clot at room temperature for about 6 hours, the clot separated from the side of the tube with a needle and then removed by centrifugation at 2000rpm for 5 minutes. The supernatant (serum) was stored in aliquots at -20°C .

3.28.3 Affinity Purification of Antiserum.

This procedure was adapted from Harlow and Lane (1988). Extract from E. coli expressing the T7 gene 10-x-rel.2 fusion protein was prepared as described in section 3.28.1. Extract containing approximately 100ug of fusion protein was divided amongst each track on a SDS polyacrylamide protein gel (see section 3.24.1). The gel was run and electroblotted onto a nitrocellulose filter (see section 3.26).

A narrow strip was cut from the edge of the filter and the fusion protein detected with the polyclonal antiserum (see section 3.27). The section of filter containing the fusion protein was then cut out, and incubated with 3ml of

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antiserum mixed with 3ml of TBS (20mM Tris.HCl pH7.6, 137mM sodium chloride) on a slow shaker for 4 hours. The filter was washed in 3 changes of TBS over 15 minutes, and then bound antibody removed by incubating the filter in 3ml of 100mM glycine pH2.5 for 5 minutes. The glycine solution (containing affinity purified antibodies) was mixed with 300ul of 1M Tris.HCl pH8, and aliquots stored at -20°C.

3.29 Sectioning and Staining of Oocytes/Embryos with Polyclonal Antiserum

Oocytes and embryos were fixed in 2% TCA for 24-48 hours at 4°C, soaked in embedding acrylamide (8.4% acrylamide; 0.0134% bis acrylamide) at 4°C for 4 hours and then embedded in polymerised acrylamide. The acrylamide blocks were frozen in isopentane over liquid nitrogen. 10um sections were cut on a Jung Frioguct, collected on 0.1% gelatin subbed slides and air dried. Sections were then fixed in cold acetone for 10 minutes, air dried and then rehydrated in PBS. 0.1% SDS was added and after a 10 minute incubation, this was removed and replaced with 1% BSA in PBS for 30 minutes at room temperature to block non-specific binding. The BSA solution was then replaced with the primary antibody (diluted 1:50 in PBS) and the sections incubated for 30 minutes at room temperature. After washing three times with PBS, for 5 minutes each, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1:50 with PBS for 30 minutes. After a repeated

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washing in PBS, the sections were mounted in a 1:1 solution of PBS/glycerol. Stained sections were viewed with a Nikon optiphot lens and the images were recorded on ASA160 Ektachrome film.

3.30 Paraffin Wax Sectioning.

3.30.1 Fixation and Embedding.

Oocytes and embryos were fixed in Bouin's fixative (75% saturated solution of picric acid, 25% (v/v) formalin (37% solution of formaldehyde) and 5% (v/v) glacial acetic acid) at 4°C overnight. Fixed samples were incubated in 70% ethanol for 1 hour, which was then drawn off and replaced with 90% ethanol and incubated for 1 more hour. Samples were washed in 2 changes of ethanol for 1 hour each. The ethanol was replaced with 2 changes of histoplast (xylene-substitute) of 1 hour each. The histoplast was then replaced with 50% xylene/50% paraplast wax and incubated overnight at 60°C. The xylene/paraplast was then replaced with 100% paraplast and the samples incubated at 60°C for 3 hours. Oocytes and embryos were embedded in paraplast in a watch-glass smeared with glycerol. After the wax had set, the whole assembly was immersed in chilled water to allow the wax blocks to float out of the watch glasses.

3.30.2 Sectioning.

Wax blocks were mounted in a microtome, whose blade was set at an angle of 16°. Sections were cut at a thickness of

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8 microns and placed on a subbed slide (i.e. slides that had been dipped into a solution of gelatin (0.5%, Sigma G-2500 porcine "300 bloom") plus chrome alum (0.05%)).

3.30.3 Dewaxing and Staining.

Slides were dewaxed by placing them in histoplast for 2 hours and then in fresh histoplast for 10 minutes. They were then washed in 100% ethanol for 10 minutes, 90% ethanol for 10 minutes and then 70% ethanol for 10 minutes. Sections were stained in Mayer's haematoxylin (prepared by dissolving 1g of Haematoxylin in 1000ml of distilled water. Then 50g of Potash alum is added, followed by 0.2g of sodium iodate and the mixture shaken. 50g of chloral hydrate and 1g of citric acid is then added and the solution stored) for 30 minutes. Sections were washed in alkaline tap water, with several changes, for 5 minutes. Sections were then immersed in 70% ethanol, 0.1% light green for 20 minutes, which was then replaced with 90% ethanol and left for 5 minutes. After two changes of 100% ethanol for 5 minutes each, followed by 2 changes of histoplast for 5 minutes each, slides were mounted in D, P, X.

3.31 Immunoprecipitation to Show Specificity of Antibody.

Extract from oocytes (equivalent to two oocytes) was mixed with 90ul of RIPA (100mM Tris-HCl (pH 7.4), 1% (v/v) Triton X-100, 0.5% (w/v) SDS, 5mM MgCl₂, 100mM KCl and 1%

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(w/v) sodium deoxycholate). Duplicates of this mixture were incubated, with shaking, for 1-2 hours at 4°C. 20ul of washed S. aureus protein A (which was washed by spinning down the required volume of S. aureus protein A and resuspending in an equal volume of RIPA; S. aureus was washed to remove free protein A because it can detach from cells and will therefore attach to, but not precipitate an antibody) was added to the samples which were then incubated (with vigorous shaking to avoid the bacteria settling out) for a further 1-2 hours at 4°C. The samples were then microfuged for 5 minutes and the supernatants from each duplicate sample taken. The pellet contained non-specifically precipitated protein. 1-5ul of pre-immune serum was added to one of the samples and 1-5ul of immune serum was added to the other sample. All samples were incubated, with vigorous shaking, at 4°C for at least 4 hours. 20ul of washed S. aureus protein A was added to each sample and the mixtures incubated for a further 2-3 hours, with vigorous shaking. The samples were then microfuged for 5 minutes and the pellets resuspended in 100ul of RIPA. This latter step was repeated twice. Finally, the pellets were resuspended in 40ul of 1x SDS gel loading buffer. The samples were heated at 96°C for 3 minutes and analysed by SDS-PAGE (see section 3.24)

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3.32 Methods for Radiolabelling DNA and RNA.

3.32.1 Nick Translation.

DNA was labelled by nick translation for use as probes in Southern and Northern blotting and Library screens. A 20ul reaction in 1x NTB (50mM Tris.HCl pH7.2, 10mM MgSO₄, 0.1mM DTT, 50ug/ml BSA) contained 100ng of DNA, 5mM 'cold' dNTPs (dATP, dCTP, dTTP), 40uCi of α -³²P-dGTP, 1ng of deoxyribonuclease I (Sigma) and 10 units of E. coli DNA polymerase I (Amersham). The reaction was allowed to proceed at 14°C for 3 hours after which 0.2ml of NaE was added. The mixture was run through a 10ml sephadex G-50 gel filtration column in TE. The higher molecular weight labelled DNA passes through this column faster than unincorporated nucleotides, allowing separation of the labelled DNA from unincorporated nucleotides. Labelled DNA (first peak of radioactivity) was collected from the column, heated at 96°C for 3 minutes, cooled on ice, and then added to hybridisation buffer.

3.32.2 PCR Prepared Probes.

Single-stranded probes were also made for use in Southern and Northern blotting, by amplifying DNA fragments using 'unidirectional' PCR. A standard PCR 100ul reaction (see section 3.12.2) was set up, except using only one primer, 20ng of template DNA and 30uCi of α -³²P-dGTP replaced unlabelled dGTP. 10 cycles of the following programme were run; 94°C 1.5', 50°C 1' and 72°C 9.9'. The labelled PCR

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product was precipitated with ethanol and the recovered pellet dissolved in 20ul of TE.

3.32.3 End-Labeling DNA with γ -³²P-ATP and T4 Polynucleotide Kinase.

Single stranded oligonucleotides (for use in in primer extension assays) and duplex oligonucleotides (for use in band shift assays) were labelled using this method. Synthetic oligonucleotides are supplied dephosphorylated at the 5' end, and can therefore be directly labelled by this method. Labelling was carried out in a 15ul reaction in 1x C buffer (66mM Tris.HCl pH7.6, 6.6mM MgCl₂, 10mM DTT) containing 4 to 10ng of oligonucleotide, 60 to 100uCi of γ -³²P-ATP and 20 units of T4 polynucleotide kinase. The reaction was incubated at 37°C for 1 hour after which 0.2ml of NaE was added. The mixture was run through a Sephadex G-50 gel filtration column in TE, in order to separate labelled oligo from unincorporated nucleotide. Fractions containing labelled DNA were collected and DNA precipitated by the addition of sodium acetate (pH6.5) to a final concentration of 0.3M and 2 volumes of ethanol. DNA was recovered by microfugation for 10 minutes, and resuspended in TE buffer.

Restriction fragments of DNA were dephosphorylated at the 5' end with calf intestinal alkaline phosphatase (essentially as described in section 3.12.3), and then labelled as described above.

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3.32.4 Labelling DNA Fragments by End-Filling.

This method was used to radiolabel DNA digested with restriction enzymes generating 5' overhangs. Up to 1 µg of restricted DNA was labelled by filling in the overhang with a nucleotide mix containing one radio-labelled nucleotide. A reaction in 1x TM buffer (10mM Tris.HCl pH7.5, 5mM MgCl₂) containing DNA, 40µCi of one α-³²P-dNTP, the other 3 'cold' dNTPs (1mM) and 10 units of the Klenow fragment of DNA polymerase I, was incubated at room temperature for 30 minutes. Labelled DNA was separated from unincorporated nucleotides by sephadex G50 gel filtration, and recovered by ethanol precipitation. This method was used to make labelled DNA markers (lambda DNA cut with Hind III and EcoR I, and pBR322 cut with Hpa II).

3.33 Preparation of Protein Extract for Band Shift Assays.

The following procedure was used to prepare protein extracts for use in band shift assays (see section 3.34), and extracts prepared in the same way were also used for SDS-protein acrylamide gel electrophoresis. All extracts were made in extract buffer: 50mM Tris-HCl pH8, 50mM KCl, 0.1mM EDTA, 5mM MgCl₂, 10µg/ml aprotinin (Boehringer), 10µg/ml leupeptin (Boehringer), 25% glycerol.

3.33.1 From Xenopus laevis Oocytes, Eggs and Embryos.

Oocytes and embryos were washed on ice in several volumes of extract wash buffer (extract buffer except with

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distilled water replacing glycerol). Samples were homogenised on ice in 5ul per embryo/oocyte of extract buffer and then microfuged at 4°C for 10 minutes. The supernatant was removed and microfuged for a further 2 minutes and the resultant supernatant snap frozen in liquid nitrogen. Enucleated oocyte extract was prepared in the same way from oocytes from which the nuclei had been removed by manual dissection (the animal pole of the oocyte was pierced with the tip of a fine needle, and the oocyte gently squeezed with a pair of watchmakers forceps to expel the nucleus with little loss of cytoplasm). Isolated nuclei were picked up with a Gilson pipette in 5ul of 1x BX (the medium in which oocytes were incubated), glycerol added to 25% (v/v), BSA added to 50ug/ml and the extract snap frozen in liquid nitrogen.

3.33.2 From E. coli Expressing Fusion Proteins. -

E. coli BL21 (DE3) transfected with fusion protein constructs were grown and induced as described in section 3.28.1, pelleted and resuspended in 0.01 volumes of extract buffer. The suspended cells were sonicated on ice, microfuged at 4°C for 10 minutes and the supernatant snap frozen in liquid nitrogen.

3.34 Band shift assays.

3.34.1 Preparation of probes.

Complementary synthetic oligonucleotides were mixed at a

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final concentration of 1mg/ml in 1x medium salt restriction digest buffer (10mM Tris.HCl pH7.4, 10mM MgSO₄, 50mM NaCl, 1mM DTT), the tube placed in 1 litre of water at 85°C and left until the water had cooled to room temperature.

Annealed oligonucleotides were then diluted to a concentration of 2ug/ml in TE buffer. 10ng of duplex oligonucleotide was end labelled with 60uCi of γ -³²P-ATP and T4 polynucleotide kinase (oligonucleotides are supplied dephosphorylated) as described in section 3.32.3.

3.34.2 Assays.

25ul binding reactions were carried out on ice for 20 minutes in binding buffer (45mM KCl, 15mM HEPES pH7.9, 5mM spermidine, 1mM MgCl₂, 1mM dithiothreitol, 0.5mM PMSF, 0.1mM EDTA, 7% glycerol) containing 2ug of pAT153, 1ug of salmon sperm DNA, 20ng of non-specific duplex oligonucleotide (ACAGACCGAAGCTTAGCT), 0.5ng of duplex oligonucleotide probe, up to 5ul of protein extract (see section 3.33) and (when added) 1ul of antiserum. For competition analysis the non-specific duplex oligonucleotide probe was replaced by 20ng of specific duplex oligonucleotide.

The reactions were electrophoresed on a 5% polyacrylamide gel (29:1 bis) in 1/4x TBE (22.5mM Tris-borate pH8, 0.5mM EDTA) at 200 volts and 4°C for 2 hours. The gel was fixed in 10% (v/v) glacial acetic acid, 10% (v/v) ethanol, dried

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on a vacuum drier at 80°C and exposed to X-ray film with an intensifying screen at -70°C.

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RESULTS AND DISCUSSION.

Chapter 4.

Isolation of X.laevis rel cDNA clones from an oocyte library.

Introduction.

The aim of cDNA library screening was to isolate Xenopus homologues of the Drosophila dorso-ventral polarity gene, dorsal (see Introduction section 1.2). A dorsal cDNA probe detected homologous sequences in the X. laevis genome, when used at low stringency on a Southern blot of restriction enzyme-digested X. laevis genomic DNA (data not shown). The result led me to screen an oocyte cDNA library (in lambda ZAP) with the dorsal cDNA as a probe. An oocyte library was selected since in Drosophila, dorsal is a maternal-effect gene. Sequence analysis of positive clones isolated from this library revealed that hybridisation had been a result of extensive stretches of CAG repeats. In dorsal, these repeats encode a glutamine-rich region. Apart from these sequences, the clones isolated showed no homology to dorsal or other rel family proteins (data not shown). In view of these problems and the fact that frogs have a closer evolutionary relationship to chickens than to flies, the chicken-derived relative of dorsal, v-rel was selected for use as a probe. V-rel, which shares 47% amino acid identity (or 60% if conservative changes are taken into account) to

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dorsal within the conserved N-terminal region, was used as a probe (this probe lacked any CAG repeats). The 1.0kb middle v-rel (gift of Thomas Gilmore and Howard Temin) is obtained by an Eco RI digestion of pUC12-REV-T3 (see Chen et al., 1981), which contains 1.9kb of v-rel sequence (see figure 7). The pBR322 plasmid containing middle v-rel, referred to as pTG7, was digested with Eco RI and Bam HI to release 550bp and 450bp fragments of DNA, which were subsequently resolved by electrophoresis on a 0.8 % agarose gel, isolated and then radioactively labelled for use as probes. A duplicate screen was carried out, one set of filters being screened with the 550bp fragment, and one with the 450bp fragment. Given the previous problems with isolating 'false' positives, it was hoped to detect clones with homology to both these probes. However, no such clones were isolated, and in fact only the 550bp N-terminal fragment (containing about two thirds of the N-terminal region conserved between rel family members) detected homologous clones. This result was not surprising since at the time of screening, a family of rel proteins was emerging, whose members all shared homology in the N-terminal half, but were completely divergent in their C-terminal half. The 450bp v-rel fragment (derived from turkey c-rel) does not share any homology with c-rel (except chicken c-rel) or other rel family members.

Figure 7.

Restriction map of v-rel (given by T. Gilmore and H. Temin), showing the fragments derived from middle v-rel, V550 and V450, that were used as probes in screening the lambda ZAP matured oocyte cDNA library.

coding region

Rel family homology

XbaI

PstI

EcoRI

BamHI

EcoRI

PstI

v-rel

REV-T3

340bp

pTG7

BamHI

middle v-rel

1 kb

100bp

550bp

450bp

Probe :

V550

V450

100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000

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4.1 Library screening.

The X. laevis matured oocyte cDNA library in lambda ZAP (a gift of Dr. John Shuttleworth) was screened with chicken v-rel probes, as described above. This library was originally obtained from total RNA prepared by the SDS-proteinase K method from progesterone matured X. laevis oocytes. Poly A⁺ RNA was prepared by two rounds of oligo dT cellulose selection and cDNA was synthesised using AMV reverse transcriptase. Eco RI linkers had been added to the cDNA, and the linkered cDNAs ligated into lambda ZAP, after the vector had been digested with Eco RI and dephosphorylated with CIAP. Phage DNA had then been packaged using 'Gigapack gold' (Stratagene). The layer of follicle cells which surrounds the oocytes had not been removed prior to the preparation of the RNA, therefore cDNAs from these cells were presumably represented in this library. The library contained 4×10^5 independent clones, with inserts in the size range 1 to 2.50 kb (as assessed from 10 randomly picked plaques). The aliquot of amplified phage stock that I received had a titre of 1×10^{10} pfu/ml.

Screening of the library was carried out as described in the Methods section 3.19. Four times the library size, that is, a total of 1.6×10^6 phage from the oocyte cDNA library, was screened with the v-rel probes. Initially the original and replica filters from each plate were probed with the 550 and 450bp probe respectively. Three replica possible positive plugs were taken from the first screen. The clones

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showed a strong reaction to the 550bp probe, and what appeared to be a trace of reaction to the 450bp probe. After the second screen the plaques were shown to hybridise only to the 550bp v-rel probe. Since no genuine positive plaques were found to hybridise to both probes, the subsequent rounds of rescreening of the three replica positive plugs were carried out using only the 550bp probe for both first and second plaque lifts. After completion of the third screen, an example of which is shown in figure 8, single positive plaques were obtained. One of the three positives was missed on rescreening. The two remaining clones were purified by plating-out and picking single plaques, without rescreening.

4.2 Automatic excision of positive rel cDNA clones from the phage vector.

The two positive cDNA clones were converted to the plasmid form, in pBluescript SK-, by automatic excision from the phage vector, according to the Stratagene manual (see Methods section 3.20). Two independent ampicillin-resistant colonies were picked for both clones since gross rearrangement can occur in the process of excision (pers. comm J. Shuttleworth). Miniprep DNA prepared from these duplicate colonies was digested with Eco RI and Hind III. Since these digests gave identical products, there was no evidence that rearrangement had occurred and a single colony representing each clone was selected for further

Figure 8.

A third round screen of a positive cDNA clone from the X. laevis cDNA library.

a) and b) are replica filters from the third round screening (where phages from a plug, obtained in the second screen, were plated out at under 100 plaques per plate onto E. coli BB4 and then plaque lifts were taken) of a matured oocyte cDNA library. These filters were hybridised to 100ng of a 550 bp Bam HI-Eco RI fragment of middle v-rel (V550), radioactively labelled by nick translation. Both filters were hybridised in a solution of: 43% deionised formamide, 6x SSC, 5x Denhardt's, 50mM NaPO₄ (pH 7.0), 0.1% SDS and 100ug/ml tRNA at 37°C for 12 hours. The filters were washed in 2x SSC, 0.1% SDS at 60°C. Plugs containing a single replica positive were taken following this third round screen.

a) Autoradiograph of the 'first lift' filter probed with V550.

b) Autoradiograph of the 'second lift' (replica filter), probed with V550.

A



B



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analysis. Eco RI digests released the insert from both clones as a single band. One clone (J) had an insert size of 2.0 kb and the other clone (A) an insert size of 1.8kb. In order to ascertain whether one of these clones (A) was a truncated version of the other (J), detailed restriction analysis was carried out.

4.3 Restriction analysis of clones A and J.

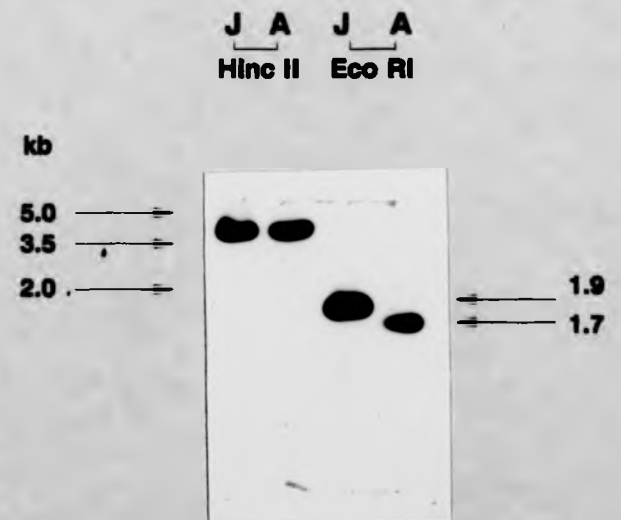
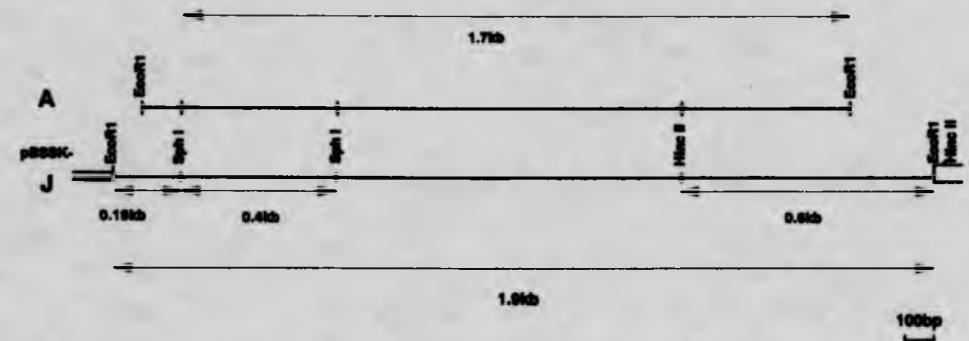
Restriction analysis was performed on both the A and J clones to give an indication of the relationship between the clones and to work out a strategy for sequencing. A map is shown in figure 9a. All the restriction sites that were identified in clone A were also present in clone J, thus these two clones were likely to be closely related. The restriction enzyme map shown in figure 9a was determined from single and double restriction enzyme digests. Both clones had a single site for Pst I and Hinc II. There was a notable lack of useful cloning sites (i.e those commonly found in sequencing vector polylinkers), including Hind III, Kpn I, Bgl II, Sal I, Sac I and Bam HI. These sites are uniquely present in the polylinker of pBluescript and were used in double digests with sites present in the clones, to determine the position of the internal sites. The conservation of the sites, as mentioned above, between the two clones indicated that they may be derived from the same message, clone A being shorter at both ends. A and J were digested with Hinc II and Eco RI and the products were

Figure 9.

The Hinc II fragment from J and A does not hybridise with V550, indicating that the Hinc II site is closer to the 3' end.

a) Restriction map of A and J. These sites were mapped prior to the sequencing of these clones.

b) The two positive clones, A and J, were digested with Eco RI and Hinc II and the digests resolved on a 0.8% agarose gel. The products were transferred to nitrocellulose by Southern blotting and hybridised to a radioactively labelled V550 probe. The hybridisation conditions were the same as for the library screening (refer to figure 8).



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resolved by agarose gel electrophoresis, Southern blotted and hybridised to radioactively labelled v-rel (550bp probe). The autoradiograph of this blot is shown in figure 9b. For both the A and J clone, there was no hybridisation of v-rel to the smaller Hinc II fragment, and the larger fragment in both clones showed homology to v-rel, which supported the interpretation that the two clones were derived from the same message. The rel homologous region was predicted to be at the 5' end of the clone (based on sequence comparisons among other rel family members) and therefore the 5' end was tentatively assigned to the end that hybridised, i.e the end furthest from the Hinc II site. Since there was a lack of restriction sites that were useful for generating fragments for subcloning into M13, it would have been difficult to obtain the complete sequence by the strategy of making subclones in M13. Consequently, it was decided to sequence the clones using a exonuclease III deletion series. This is described in the next section.

Chapter 5.

Sequence analysis of rel homologues.

5.1 Sequencing of clones A and J.

Sequencing in all cases was carried out by the dideoxy chain termination method. Both clones A and J were completely sequenced by a combination of double- and single-stranded sequencing using the universal and reverse M13 primers (both 17mers). The strategy used is outlined in figure 10a.

Due to the lack of useful restriction sites, a double-stranded nested deletion series, using exonuclease III, was created for both A and J clones. Such a unidirectional deletion series produces deletions in DNA fragments cloned in plasmid vectors (or M13 vectors), starting with double-stranded DNA. This strategy enables the complete sequence of a large fragment of DNA to be determined using the same primer (see figure 11). The nested deletions extend progressively further into one end of the cloned fragment, leaving the universal primer site in tact. Selected subclones from the nested deletion series, can then be used to sequence progressively further into the cloned fragment, in steps of 200-250 nucleotides. This method is based on the controlled digestion of DNA with the 3'-exonuclease, *exo III*. This digestion can be made unidirectional since *exo III* attacks some types of 3' end and not others; blunt and 5'-overhanging ends are susceptible, whereas 3'-overhanging ends are resistant, provided the overhang is

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Figure 10.

Strategy used in sequencing J and A and detailed restriction map determined from sequencing.

a) Sequence strategy for J and A. Dotted arrows refer to areas of the clones sequenced by single-stranded sequencing of M13 subclones. Arrows below these, refer to overlapping regions of the clones which were sequenced by double-stranded sequencing of the subclones obtained from the exonuclease III deletion series.

b) A detailed restriction map showing restriction sites that were used in subsequent cloning procedures (use as reference to later chapters).

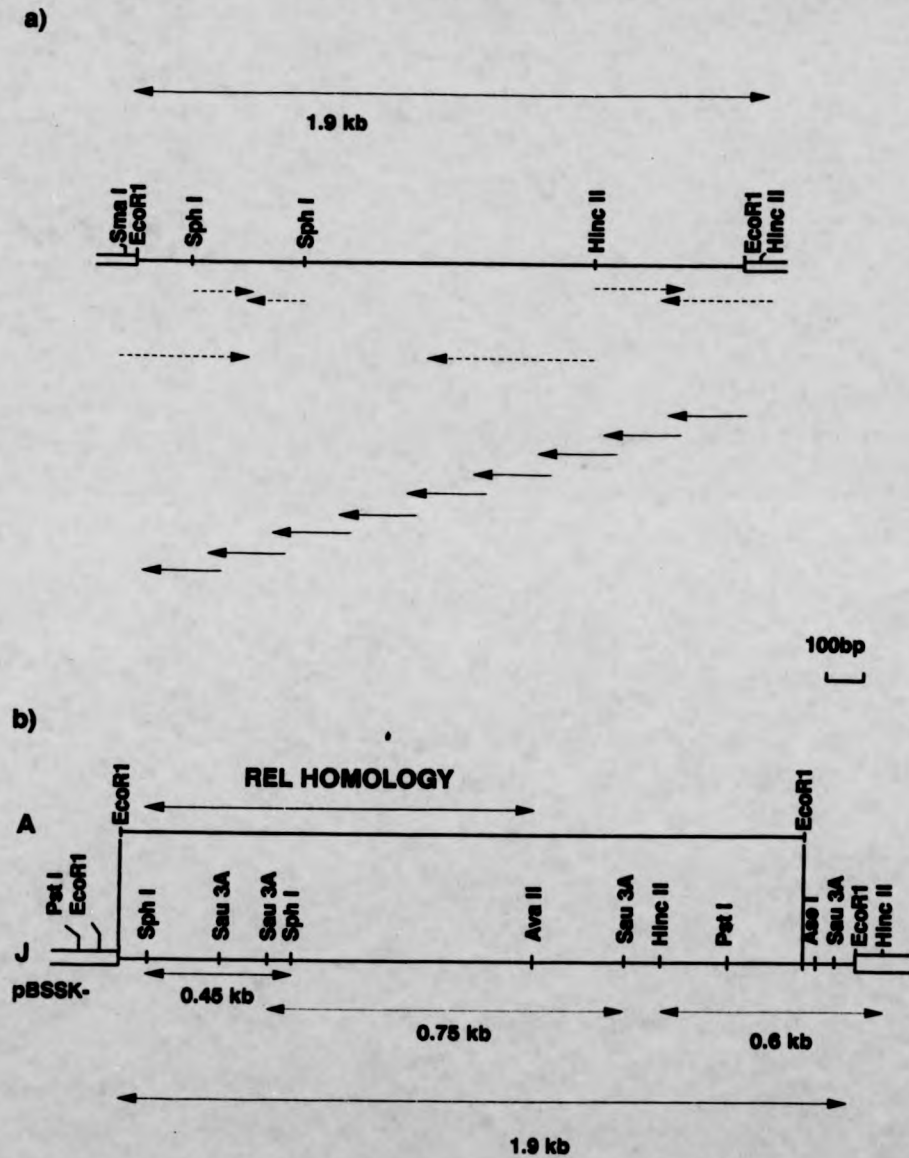


Figure 11.

Outline of strategy used in creating exonuclease III deletion series.

3 μ g of A and J were digested with Kpn I and Hind III, which only cut in the pBluescript polylinker. Hind III produces a 5' overhang, which is susceptible to exonuclease III digestion and Kpn I produces a 3' overhang which is not susceptible to attack. Exonuclease III digested this exposed strand of DNA and deleted from the 3' end of A and J. S1 nuclease is used to digest the remaining single strand of DNA and the deleted clones are religated to create plasmids containing progressively smaller amounts of J or A, which can then be sequenced by double-stranded sequencing.

The solid line represents insert cDNA; the broken line represents pBluescript vector.

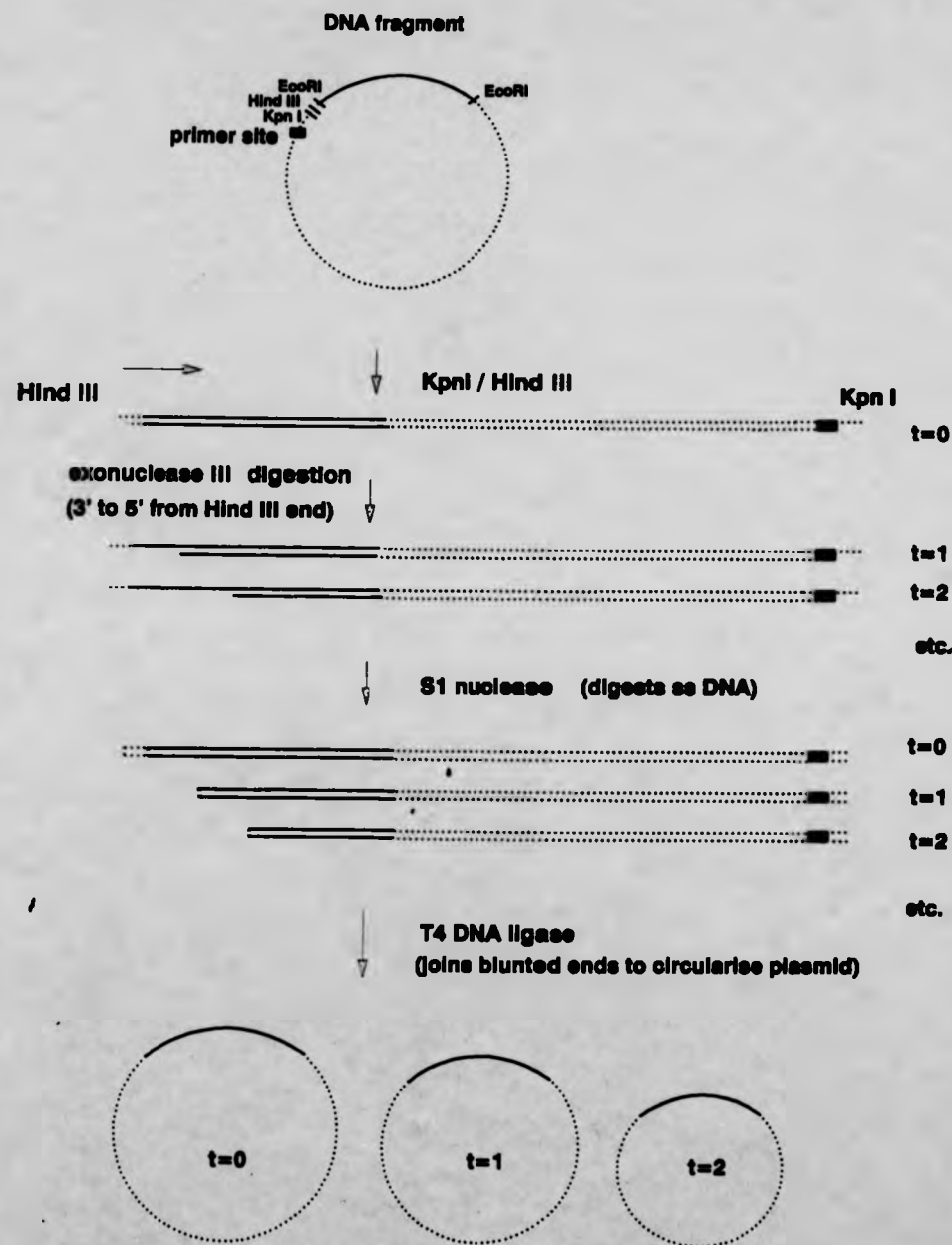
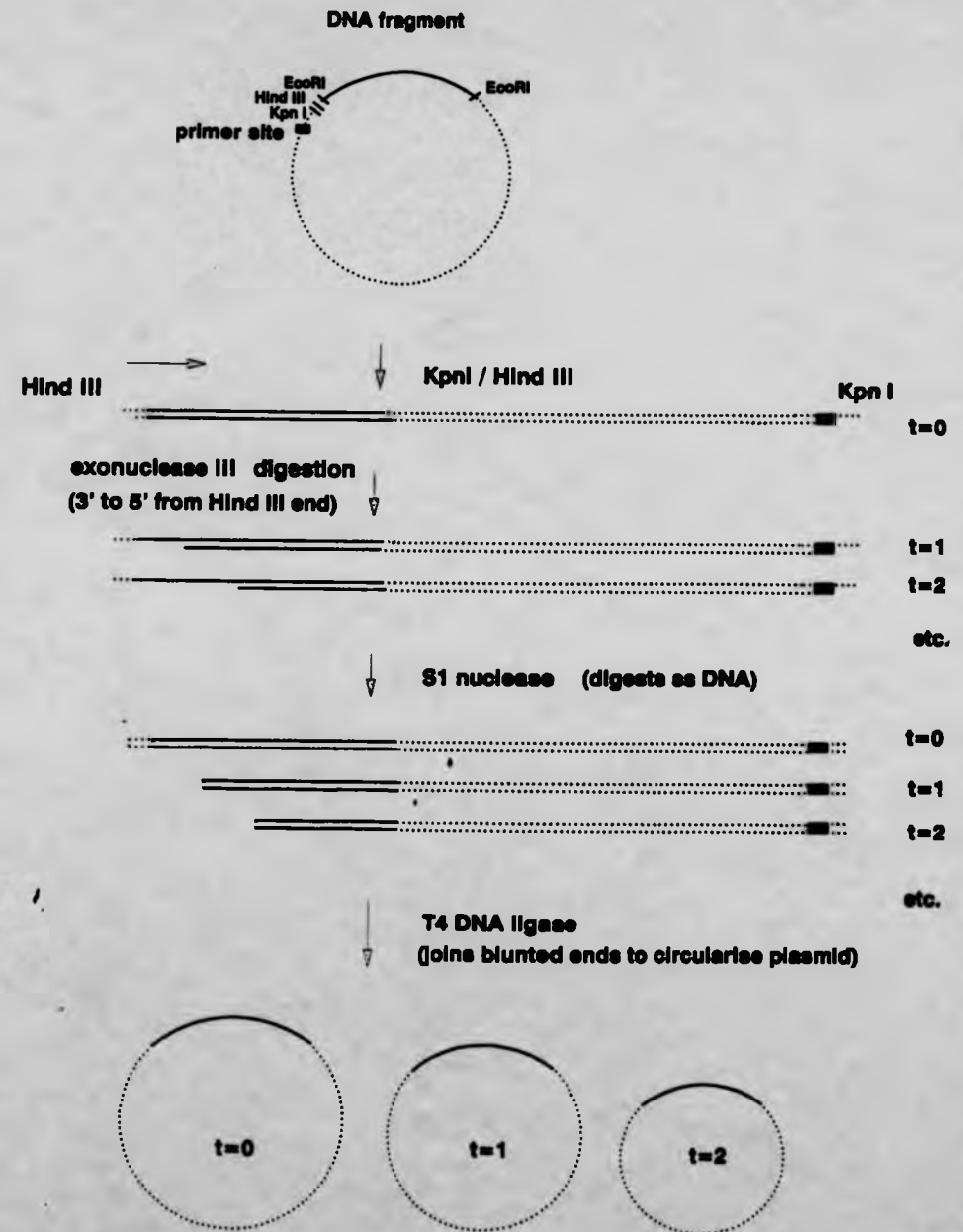


Figure 11.

Outline of strategy used in creating exonuclease III deletion series.

3 μ g of A and J were digested with *Kpn* I and *Hind* III, which only cut in the pBluescript polylinker. *Hind* III produces a 5' overhang, which is susceptible to exonuclease III digestion and *Kpn* I produces a 3' overhang which is not susceptible to attack. Exonuclease III digested this exposed strand of DNA and deleted from the 3' end of A and J. S1 nuclease is used to digest the remaining single strand of DNA and the deleted clones are religated to create plasmids containing progressively smaller amounts of J or A, which can then be sequenced by double-stranded sequencing.

The solid line represents insert cDNA; the broken line represents pBluescript vector.



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three or more bases in length. Exo III removes nucleotides (3' to 5') from one strand of the target DNA, creating a single-stranded region which can be removed using S1 nuclease. The plasmid is then recircularised using T4 DNA ligase.

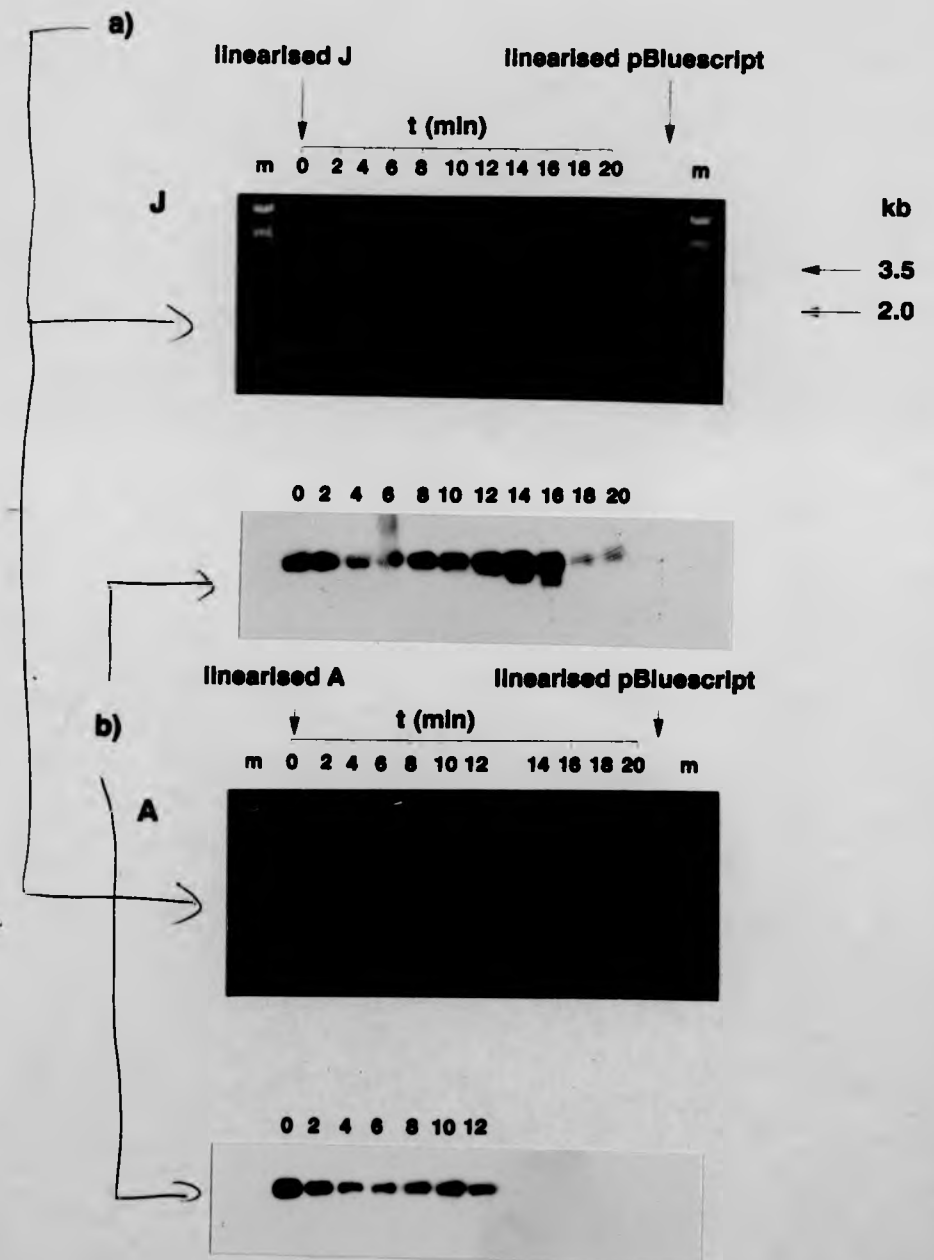
A and J pBSSK⁻ clones were therefore digested with Kpn I to produce a protected 3'-overhang and Hind III to produce a 5'-overhang susceptible to digestion with exonuclease III. Kpn I and Hind III sites occur in the pBluescript polylinker after the Eco RI site, as shown in figure 11. The Eco RI-inserted fragment was therefore deleted from the C-terminal to the N-terminal end. The exonuclease deletion reaction was quantified and it was found that 200 units of exonuclease III at 35°C resulted in a deletion rate of approximately 100 bp a minute. Samples were taken every 2 minutes over a period of 20 minutes in order to obtain ten clones, each containing about 200bp less of the inserted fragment. Aliquots of these samples were resolved on a 0.8% agarose gel, Southern blotted and hybridised to v-rel. A photograph of this gel and of the autoradiograph is shown in figure 12. The stepwise reductions of the insert led to the last time point approaching the size (about 200bp larger) of linearised pBluescript. Again, both clones hybridised to V550 in a particular region of the clone; the signal was lost at time point $t > 14$ minutes. This hybridising end was assumed to be the 5' end on the basis of sequence comparison among rel

Figure 12.

Preparation of an exonuclease III nested deletion series.

a) 3 μ g of J and A were each digested with 30 units of Kpn I and then 30 units of Hind III. The digest was precipitated and the resuspended DNA subjected to 200 units of exonuclease III. The reaction was carried out at 35°C over a period of twenty minutes. 6 μ l samples were taken every two minutes and half of the volume of these aliquots (about 150ng) were resolved by electrophoresis on a 0.8% agarose gel. The deletion rate was about 100 bp/minute. Linearised J/A and linearised pBluescript were electrophoresed either side of the deletion series. The markers, indicated by m, are lambda DNA digested with Eco RI and Hind III.

b) About 10ng of the remaining time point samples were resolved on a 0.8% agarose gel and Southern blotted to a nitrocellulose filter. The blot was hybridised to radioactively labelled V550; hybridisation and washing conditions were as described for figure 8.



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family members.

Ideally, double-stranded plasmid sequencing of the nested deletion series would eliminate the need for subcloning into M13 vectors. However, where sequence obtained from double-stranded sequencing was ambiguous, some M13 subclones were made and both strands were sequenced. These ambiguous areas of sequence were clarified by making three M13 subclones. A 450bp fragment isolated by digesting A and J with Sph I (see figure 10b) was subcloned into M13 in both orientations and single-stranded template DNA from these clones was sequenced. Sequence from the two orientations overlapped, providing the complete sequence of this 450bp fragment. A and J were also digested with Hinc II and Sma I; there is a Hinc II site about 600bp from the 3' end of the clones and Hinc II and Sma I sites are present in the Bluescript polylinker, either side of the Eco RI-inserted fragment. This double digest produced a 600bp Hinc II fragment and a 1300bp Hinc II-Sma I fragment. Hinc II and Sma I digests produce compatible blunt ends, thus both orientations of both of these fragments were subcloned into M13 that had been digested with Hinc II and dephosphorylated with CIAP. Single-stranded template DNA from these M13 subclones was sequenced by standard and extended sequencing (see Methods sections 3.22.1 and 3.22.2). Most of the Hinc II fragment and about half of the Hinc II-Sma I fragment were sequenced by this method and this allowed the remaining discrepancies, obtained from

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double-stranded sequencing of the nested deletion series, to be clarified. A and J are indeed closely related, but J is larger at both ends and there are two internal nucleotide differences.

The complete nucleotide sequence of J is shown in figure 13. The differences in the 5' and 3' ends of the A clone are indicated, as are two internal nucleotide differences (*1 and *2) between the two clones. One of these internal differences, *1 is due to an extra G residue in the J sequence (taken out of the sequence in figure 13), which causes a frame shift so that only the shorter A clone is predicted to produce a full-length protein; *2 is due to the substitution of a G residue for a C residue. These internal differences may be due to the errors produced by the reverse transcriptase (which on average causes an error of about 1 in every 2000 bp) in the preparation of the library. However, they could be polymorphisms in a heterozygous animal, or the result of the gene duplication in Xenopus laevis evolution (Xenopus laevis is tetraploid with respect to a theoretical ancestor; Bisbee et al., 1977). Translation of the longest open reading frame (ORF) from the first in frame methionine is indicated. An in-frame stop codon occurs six codons before this methionine, suggesting that the ATG indicated is the genuine translation start point. The sequence around this ATG - GAACATGGAT - fits with Kozak's rules for a favourable context for the initiation of translation (Kozak, 1989).

Figure 14.

Comparison of protein sequence of rel family members.

The comparison between these proteins was carried out using Clustal analysis (programmes 1, 2, and 3; Higgins and Sharp, 1988). Clustal 4 was not used since it aligns proteins from end to end, which was not applicable in this case since these eight proteins share homology mainly in the N-terminal end. Gaps to allow for maximum homology are shown by -; identical amino acids for all nine proteins are shown by *; conservative amino acid changes in all proteins are shown by a dot.

Gap penalty = 4 Cut-off = 10
Variable gap penalty = F Window = 10 Filtering = 2.5
* :-> Exact match across all seqs.; :-> Conservative matches across all.

```
xrel-1 -----MDGFHWTDIVSSMPPSIPVEIIEQPKQRGM
hup65 -----MDDLFLIFPSEPAQASGPVVEIIEQPKQRGM
kbf1 ----MAEDDPYLGRPEQMFLDPSLTHITFNPEVFPQMALPTADGPLYLQILEQPKQRGF
hup49 -----MESCYNPGLDGIIEYDDFKLNSIVEPK-----EPAPETADGPLYLVEQPKQRGM
huc-rel -----MAGAYNPYIEIIEQPRQRGM
moc-rel -----MAGAYNPYIEIIEQPRQRGM
tuv-rel -----MDFLTNLRFTGISEPYIEIIEQPRQRGT
chc-rel -----MAGISEPYIEIIEQPRQRGM
dorsal MFPNQNGAAPGQGPVVDGQQLNLYNGLPAQQQQQLAQTSTKVRKPKYVKITEQFAGKAL
* . * * * *
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xrel-1 RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
hup65 RFRYKCEGRSAGSIPGERSTDTTKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHLGV
kbf1 RFRYVCEGPGSHGGLPGASSEKKNKSYVQVKICNYVGPAAKVIIVQLVTNGKNIHLHAHSLVG
hup49 RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
huc-rel RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
moc-rel RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
tuv-rel RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
chc-rel RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
dorsal RFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGPARIIRISLVTKDSPHKPHPHLGV
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xrel-1 KD-CKDGYEAEELSPDRSIHSPQNLGIQCVKREVEDAVAHRIRTNN-----
hup65 KD-CRDGYEADLCPDRSIHSPQNLGIQCVKRRDLEQAIISQRIQTNN-----
kbf1 KHC-EDGICTVTAGPKDMVVGFPANLGIHLVTKKVFETLEARMTAEACIRGYNPGLLVHPD
hup49 KGCSELGICAVSVGPKDMTAQFNGLVHLVTKKMMGTMIQKLRQRRLRSRPG-----
huc-rel KD-CRDGYEAEFGQERRPLF-----
moc-rel KD-CRDGYEAEFGPERRPLFPQNLGIRCVKKEVKGAIILRISAGI-----
tuv-rel KG-CRDGYEAEFGPERQVLSFQNLGIQCVKRRDLKESISLRISKKI-----
chc-rel KD-CRDGYEAEFGPERRVLSFQNLGIQCVKRRDLKESISLRISKKI-----
dorsal KEGCKKGVCTLEINSETMRAVFSNLGIQCVKRRDIEAALKAREIRV-----
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xrel-1 -----NPFNVSPPEELKADY--DLNTVCLCFQVFIPOQAAGRMLP-LPF
hup65 -----NPFHVPIEBQRGDY--DLNAVRLCFQVTV-RDPAGRPLL-LTP
kbf1 LAYLQAEQGGDRQLGDREKELIROAALQQTKEMDLSVVRIMFTAFPLDSTGFSFTRR-LFP
hup49 -----LTEAEQRELEQAEKELKVMDLIVRLRFSAFRLASDGSFSLP-LKP
huc-rel -----DCDLNVVRLCFQVFLPDEHGNTLA-LPP
moc-rel -----NPFNVGEQQLLDIEDCDLNVVRCVPMFFLPDEGNTLA-LPP
tuv-rel -----NPFNVPEEQQLHNIIDEYDLNVVRLCFQAFPLPDEHGNTLA-LPP
chc-rel -----NPFNVPEEQQLHNIIDEYDLNVVRLCFQAFPLPDEHGNTLA-LPP
dorsal -----DPFKTGFSHRFQPSIDLNSVRLCFQVFMESQKGRFSTPLPP
* * * *
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xrel-1 VVSQPIYDNRAPNTAELKIC-RVNKNKSGSCLGGDEIFLLCDKQVK-DIEVIFGLGN----
hup65 VLSHPIDNRAPNTAELKIC-RVNKNKSGSCLGGDEIFLLCDKQVKEDIIEVYFTGPG----
kbf1 VVSDAIYDSKAPNASNLKIV-RMDRTAGCVTGGEEIYLLCDKQVKDDIIEVRYEENG
hup49 VTSQPIHDSKSPGASNLKIS-RMDRTAGSVRGGEDEVYLLCDKQVKDDIIEVRYEENG
huc-rel VVSNPIYDNRAPNTAELRIC-RVNKNKSGSVRGGEDEIFLLCDKQVKDDIIEVRFVLDN----
moc-rel IVSNPIYDNRAPNTAELRILARVNKNKSGSVRGGEDEIFLLCDKQVKDDIIEVRFVLDN----
tuv-rel LISNPIYDNRAPNTAELRIC-RVNKNKSGSVRGGEDEIFLLCDKQVKDDIIEVRFVLDN----
chc-rel LISNPIYDNRAPNTAELRIC-RVNKNKSGSVRGGEDEIFLLCDKQVKDDIIEVRFVLDN----
dorsal VVSEPIFDKAMS--DLVIC-RLCSCSATVFGNTQIILLCEVAKEDISVRFVLDN----
* * * * . * * * . * * * . * * * . * * * . * * * . * * *
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xrel-1 GGDSLTSLELDFSSLLSNMK-----
hup65 GDEFSSSIADMDFSALLSQISS-----
kbf1 LNAIHLAMMSNSLPCLLLLVAAGADVNAQEQKSGRTALHLAVEHDNISLAGCLLLEGDH
hup49 -----
huc-rel NTTVFVSQSDAFEGSDFSCADNSMINESGSPSNSTNPNSHGFFVQDSQYSGIGSMQNEQLSD
moc-rel SS-VFVSQSDAFDRSNFSCVDNGLMNEPGLSD--DANNPTFVQSSHYS-VNTLQSEQLSD
tuv-rel MGAALVSAAGMQSVSSSSIVQGTHQA----SATTASIMTPRTPGEVPPFLRQ----QVGY
chc-rel MGAANVSAADMQSASSNSIVHATHQA----SATAASIVNMETNDMCTSLNFEKYTQVLN
dorsal TAGNQADNNGPTLSNLLSPDSGQLVHINSEDOQILRLNSEDLIQSNLSIST-----

xrel-1 -----
hup65 -----
kbf1 VDSTTYDGTTPPLHIAAGRGSTRLAALLKAAGADPLVENFEPLYDLDDSWENAGEDEGVVP
hup49 -----
huc-rel SFPYEFFQV-----
moc-rel PFTYGFPKI-----
tuv-rel RS-----
chc-rel VSNHRQQLHQAPAAPPPVAAPGSTPPSSQPNLADTAVYNSFLDQEVISDSRLSTNPLQNH
dorsal -----

xrel-1 -----
hup65 -----
kbf1 GTTPLDMATSWQVFDILNGKPYEPEFTSDDLQAQDMKQLAEDVKLQYKLEIPDPDKN
hup49 -----
huc-rel -----
moc-rel -----
tuv-rel -----
chc-rel QNSLTLTDNQFYDTDGVHTDELYQSFQLDTNILQSYNH-----
dorsal -----

xrel-1 -----
hup65 -----
kbf1 WATLAQKLGILNNAFRLSPAPSKTLDNDYEVSGGTVRELVEALRQMGYTEAIEVIQAA
hup49 -----
huc-rel -----
moc-rel -----
tuv-rel -----
chc-rel -----
dorsal -----

xrel-1 -----
hup65 -----
kbf1 SSPVKTTSAHSLPLSPASTRQQIDELRDSVSDVGTGVTFRKLSFTESLTSGASLLTL
hup49 -----
huc-rel -----
moc-rel -----
tuv-rel -----
chc-rel -----
dorsal -----

xrel-1 -----
hup65 -----
kbf1 NKMPhDYGQEGPLEGKI
hup49 -----
huc-rel -----
moc-rel -----
tuv-rel -----
chc-rel -----
dorsal -----

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For a favourable context for initiation, the most important residues are a purine three bases upstream of the ATG and a G immediately after the ATG (PuXXATGG). Both clones have a poly A⁺ tail and two potential polyadenylation signals are shown. It seems that the first of these produced the shorter 3' untranslated region in clone A. It is likely that the two clones represent a full-length coding sequence.

Following completion of this section of the work, Kao and Hopwood (1991) reported the cloning of a Xenopus laevis cDNA with homology to v-rel. The coding sequence they report is identical to x-rel except for two amino acid differences. The first of these differences is the addition of a glutamate (E) residue after amino acid 221. Apart from the frame-shift, A and J differ by a single base change in the coding region. At residue 343, J has threonine (ACA) and A has arginine (AGA). The sequence reported by Kao and Hopwood (1991) has arginine at residue 343.

The next section includes sequence comparison of the two clones with v-rel and other rel-related genes and will indicate that A and J are true homologues of c-rel. The terms x-rel.1 and x-rel.2 will therefore be used to describe clones J and A respectively.

5.2 Comparison of X. laevis x-rel protein sequence with other members of the rel family.

The predicted amino acid sequence of x-rel was compared to those of other members of the NF-kB and rel families (figure 14). These members include; human p65, human KBF1(p50₂), human p49, human c-rel (p85), mouse c-rel, turkey v-rel, chicken c-rel and dorsal; see Introduction section 1.3.1. The comparison between these proteins was carried out using the Clustal sequence analysis programme (Clustal 3; Higgins and Sharp, 1988).

There is a high degree of similarity within the N-terminal halves of these proteins, whereas in the C-terminal halves the homology breaks down completely when all these proteins are compared. However, certain individual members also share homology within this region (see figure 14). X-rel is most similar to p65; it is 77.5% identical in the N-terminal region (see table 4) and 56.5% identical (not allowing for conservative changes, which increases the identity by about 10%; see table 5) when comparing the full-length proteins. This degree of similarity strongly suggests that x-rel may be the Xenopus laevis homologue of p65 (there is more evidence for this later). Mouse c-rel is most similar to human c-rel (p85), suggesting it is the murine homologue of p85; it is 77.9% identical in the N-terminal region and 64.5% identical, when comparing the full-length proteins (see table 4 and table 5). As one would expect, turkey v-rel and chicken

Table 4.

‡ Similarity between rel family members over N-terminal rel homologous region.

‡ sequences were evaluated by performing alignments over equivalent lengths of sequences, using Microgenie (Beckman).
‡ numbers in brackets indicate the ‡ similarity allowing for conservative changes.

- hup65 - Ruben et al., (1991). Science 251: 1490-1493.
- hukbfl - Kieran et al., (1991). Cell 62: 1007-1018.
- hup49 - Schmid et al., (1991). Nature 352: 733-736.
- huc-rel - Brownell et al., (1989). Oncogene 4: 935-942.
- moc-rel - Grumont and Gerondakis (1989).
Oncogene Res. 4: 1-8.
- tuv-rel - Stephens et al., (1984). Proc. Natl. Acad. Sci.
USA 80: 6229-6233.
- chc-rel - Capobianco et al., (1990).
Oncogene Res. 5: 257-265.
- dorsal - Steward (1987). Science 238: 692-694.

	x-rel	hup65	kbfl	hup49	huc-rel	moc-rel	tuv-rel	chc-rel	dorsal
x-rel									
hup65	77.5(85.1)		42.3(51.9)	43.6(54.3)	61.5(67.4)	64.6(71.5)	68.6(77.9)	67.9(76.9)	47.5(58.2)
kbfl	42.3(51.9)	43.5(51.4)		45.8(57.2)	59.5(67.8)	61.8(70.3)	64.7(73.7)	66.2(75.2)	46.2(55.4)
hup49	43.6(54.3)	43.5(51.4)	63.4(73.2)		42.2(49.2)	44.8(54.0)	45.1(54.0)	45.4(54.9)	43.7(51.5)
huc-rel	61.5(67.4)	45.8(57.2)	63.4(73.2)	42.3(52.8)		46.1(58.5)	45.9(57.0)	45.2(57.0)	43.5(53.0)
moc-rel	64.6(71.5)	59.5(67.8)	42.2(49.2)	42.3(52.8)	77.9(81.7)		73.0(78.2)	74.4(79.9)	46.1(56.2)
tuv-rel	68.6(77.9)	61.8(70.3)	44.8(54.0)	46.1(58.5)	77.9(81.7)	76.9(85.7)		79.0(88.6)	45.3(55.0)
chc-rel	67.9(76.9)	64.7(73.7)	45.1(54.0)	45.9(57.0)	73.0(78.2)	79.0(88.6)	97.9(98.6)		47.6(60.8)
dorsal	47.5(58.2)	46.2(55.4)	43.7(51.5)	43.5(53.0)	46.1(56.2)	45.3(55.0)	47.6(60.8)	47.0(60.1)	

Table 5.

% Similarity between rel family members over full-length sequence.

See legend for table 4.

	x-rel	hup65	kb1	hup49	huc-rel	moc-rel	tuv-rel	chc-rel	dorsal
x-rel									
hup65	56.5(64.6)		29.7(33.4)	35.1(44.9)	35.7(40.9)	44.2(51.7)	46.3(54.1)	45.4(53.1)	32.8(41.0)
kb1	29.7(33.4)	32.2(38.5)		35.7(45.2)	42.4(50.0)	43.6(51.7)	45.3(55.3)	45.0(52.9)	33.7(41.2)
hup49	35.1(44.9)	32.2(38.5)	50.0(58.5)		30.0(37.4)	32.1(39.2)	32.5(41.3)	30.7(40.4)	29.0(35.5)
huc-rel	35.7(40.9)	35.7(45.2)	50.0(58.5)	35.3(44.2)		38.5(49.5)	39.3(48.7)	38.6(48.6)	32.3(39.7)
moc-rel	44.2(51.7)	42.4(50.0)	30.0(37.4)	35.3(44.2)	64.5(72.2)		51.8(58.5)	50.0(57.3)	31.8(39.2)
tuv-rel	46.3(54.1)	43.6(51.7)	32.1(39.2)	38.5(49.5)	64.5(72.2)	55.4(66.1)		53.4(64.0)	30.3(39.3)
chc-rel	45.4(53.1)	45.0(52.9)	32.5(41.3)	39.3(48.7)	51.8(58.5)	55.4(66.1)	89.0(90.4)	89.0(90.4)	34.4(45.2)
dorsal	32.8(41.0)	33.7(41.2)	29.0(35.5)	32.3(39.7)	31.8(39.2)	30.3(39.3)	34.4(45.2)	33.1(43.3)	

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c-rel are very closely related; they are 89.0% identical over the whole protein. The dorsal protein, which is more diverged in the N-terminal domain than any of the other proteins, does not share any significant homology in the C-terminal half with any of these proteins. The C-terminal half of the dorsal protein bears the stamp of many Drosophila proteins in that it contains long stretches of glutamine residues. Although glutamine-rich regions have been found in some mammalian transcription factors, eg. Spl (Courey and Tjian, 1988), they are particularly common in other Drosophila proteins, eg. the products of zeste (Pirotta *et al.*, 1987), Antp (Schneuwly *et al.*, 1986) and cut (Blochlinger *et al.*, 1988).

X-rel, like the other members of the rel family, has a consensus nuclear localisation signal (KRKR) at amino acids 300-304 and a conserved site (RRPS) for phosphorylation by a cyclic AMP (cAMP) kinase at amino acids 272-275. X-rel, like p65, is very proline- and serine-rich, particularly in the C-terminal region. Overall x-rel protein contains 10.3% serine residues and 8.2% proline residues; in the C-terminal region (non-homologous half), that is, in the putative activation domain (see Introduction section 1.3.1) the proline content is 8.6% and the serine content is 14.9%.

Chapter 6.

The occurrence of dorsal/rel homologues in the X.laevis Genome and X. laevis embryonic RNA.

6.1 X. laevis genomic Southern blot.

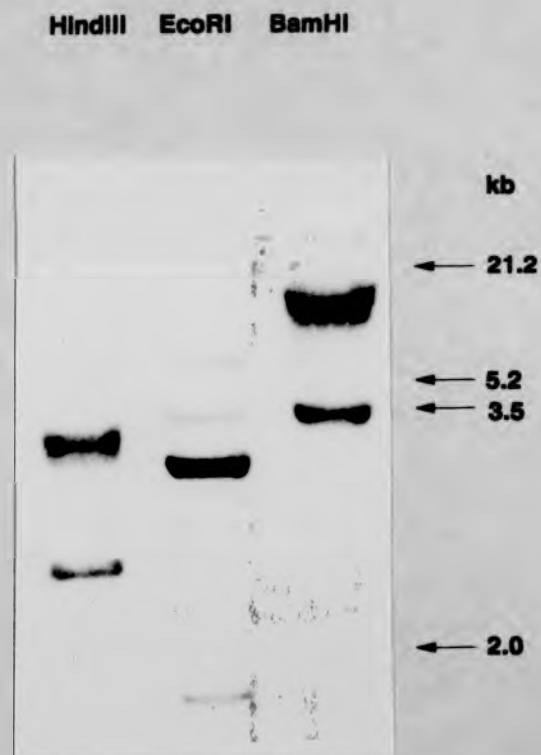
X. laevis genomic DNA was digested with the restriction endonucleases Hind III, Eco RI, and Bam HI. The digests were electrophoresed, at low voltage, on a 0.6% agarose gel and the gel was Southern blotted and hybridised to radioactively labelled x-rel.2 insert. The resultant autoradiograph is shown in figure 15. The hybridisation and washing conditions were not very stringent (0.2 xSSC, 0.1% SDS: 50°C) in order to try to detect other X. laevis rel family members.

Since the entire x-rel.2 insert was used as a probe, and most protein coding genes in eukaryotes are large with several introns, it would be expected that x-rel.2 would detect several bands in a genomic digest of X. laevis DNA as a consequence of hybridisation solely to the x-rel.2 gene. In addition, X. laevis is tetraploid with respect to a theoretical evolutionary ancestor and therefore contains two copies of each gene per haploid genome (Bisbee et al., 1977). Consequently, the hybridisation pattern observed, which revealed two strong bands, could easily be consistent, even at low stringency, with the x-rel.2 probe hybridising only to a single or duplicated x-rel.2 gene and not to related family members. The fact that higher

Figure 15.

Southern blot of *X. laevis* genomic DNA probed with *x-rel.2*

10ug of *X. laevis* genomic DNA (prepared as described in the Methods Section) was digested with 30 units of *Hind* III, *Eco* RI and *Bam* HI for 5 hours. The digests were resolved by slow electrophoresis on a 0.6% agarose gel. The gel was transferred to nitrocellulose by Southern blotting and hybridised to 100ng of *x-rel.2* cDNA radioactively labelled by nick translation. The blot was hybridised at 37°C in a solution of 6x SSC, 50% deionised formamide, 5x Denhardt's, 0.1% SDS and 100ug/ml tRNA. The filter was finally washed in 0.2x SSC, 0.1% SDS at 50°C. An autoradiograph after a 12 hour exposure to the filter is shown here. 1ug of *Eco* RI-*Hind* III digested lambda DNA was run as a marker, and the position and size of marker bands (determined by ethidium bromide staining of the gel before transfer) is shown.



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molecular weight bands show a weaker signal (most clearly seen in the Eco RI track) could be interpreted as these bands representing genes related to x-rel.2, but could equally indicate that these bands could contain less exon than the lower molecular weight bands. In conclusion, by comparison to the situation in mammals, one would expect several rel family members in Xenopus, which would be detected by a probe containing the rel homologous (RH) domain at low stringency. However, the number and size of bands detected in this Southern blot, provide no striking evidence for a family of genes, and could be consistent with the probe detecting only x-rel.2 alleles. The result of this genomic Southern also provides evidence that the clones that were isolated from the X. laevis cDNA library are not likely to be contaminants, but represent a genuine rel homologue in ovary.

6.2 X. laevis A⁺ selected Northern blot.

In order to establish the number, size and expression pattern of rel transcripts, poly A⁺ selected RNA extracted from various embryonic stages was electrophoresed on a denaturing agarose gel and Northern blotted to a piece of nitrocellulose. The filter was hybridised to a radioactively labelled x-rel cDNA probe and the resultant autoradiograph is shown in figure 16. A single species of x-rel transcript, which is about 2kb, occurs at Stage 6, but since zygotic transcription does not occur until MBT

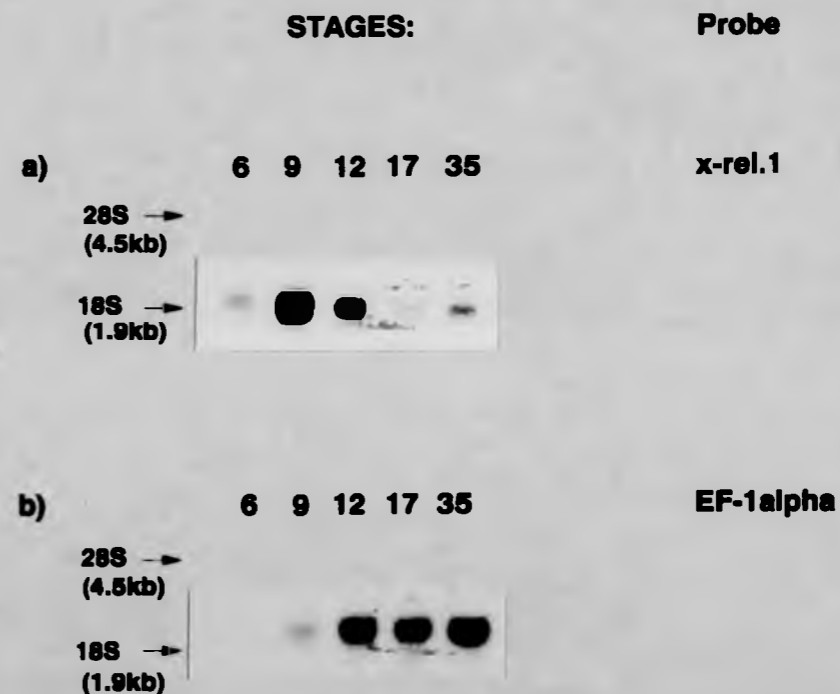
Figure 16.

Northern blot of *X. laevis* RNA probed with x-rel.2.

Total RNA was extracted from *X. laevis* embryos of the indicated stages and poly A⁺ RNA selected by one round of oligo dT cellulose chromatography. 5ug of poly A⁺ RNA for each stage was resolved on a 1.5% denaturing (formaldehyde) agarose gel, which was blotted onto nitrocellulose. The blot was hybridised at 37°C in a solution of 6x SSC, 5x Denhardt's, 50% deionised formamide, 0.1% SDS and 100ug/ml tRNA.

a) The blot was first hybridised to 100ng of x-rel.2 cDNA radioactively labelled by nick translation. The blot was washed in 0.1x SSC at 50°C. An autoradiograph after a 2 week exposure is shown. The position of the ribosomal bands is indicated (as determined by ethidium bromide staining of the gel before transfer).

b) The probe was then stripped from the filter by washing in 0.1x SSC and 0.1% SDS at 80°C and reprobed with a radioactively labelled EF-1α probe (prepared by linear, unidirectional PCR amplification of a Pst I-Sac I EF-1α 300 bp insert in pBluescript, using 200ng of T7 primer and 30uCi of α-³²P-dGTP). An autoradiograph after a 12 hour exposure is shown.



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(Stage 8 $\frac{1}{2}$; Newport and Kirschner, 1982b), x-rel transcripts must occur prior to Stage 6, and are therefore maternal. The clones were isolated from a matured oocyte (including follicle cells) lambda ZAP cDNA library and this indicates that x-rel transcripts are not solely derived from follicle cells. X-rel transcripts increase prior to the onset of gastrulation (stage 9/10) and then decrease towards the end of gastrulation (Stage 12), but are still present later in embryogenesis (Stage 35). A single band of approximately 2kb is present, as determined from its position relative to ribosomal bands detected by ethidium bromide staining. The pattern of expression is not due to, for example, degraded RNA samples or loading errors producing different amounts of RNA since ethidium bromide staining of ribosomal bands remaining after the single round of A^+ selection used, indicate that an equal amount of RNA was loaded for each sample.

The same filter was also reprobbed with the translational elongation factor, EF-1 α to ensure the quantity and quality of RNA was constant at each stage. An EF-1 α cDNA Pst I-Sac I 300bp insert in pBluescript (gift of P. Krieg) was used as a probe. Labelled single-stranded DNA was produced by linear unidirectional PCR amplification of the DNA fragment. 200ng of T7 primer and 30uCi of α - 32 P-dGTP were used to generate an anti-sense radioactive probe. The bands detected are shown in the same figure described above. Reprobing of the filter with the EF-1 α probe

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resulted in a pattern that is consistent with the data for EF-1 α transcription (Krieg *et al.*, 1989). There is a low level of a 1.7kb EF-1 α transcript at Stage 6 and about a 50x increase at gastrula. The level of this EF-1 α transcript then increases steadily in RNA isolated from late-blastula to late-neurula stage embryos. A longer exposure of the EF-1 α probed filter reveals a larger transcript (greater than 3kb) which is present in each track after gastrulation (data not shown), consistent with previous results (Krieg *et al.*, 1989). One EF-1 α transcript therefore occurs prior to, or during early gastrulation, and a separate one later in development.

Assuming the distribution of x-rel message reflects that of the protein, the fact that it is maternal is consistent with a role analagous to dorsal. X-rel protein persists until at least stage 35 and dorsal protein persists until germband extension. If x-rel were the Xenopus homologue of dorsal, one might predict that it would activate the zygotic Xenopus homologues of twist and snail, Xtwi and Xsna respectively. Xtwi RNA begins to accumulate in early gastrulae (stage 11) (Hopwood *et al.*, 1989) and Xsna is expressed strongly at the start of zygotic transcription (Sargent and Bennett, 1990). X-rel, whose expression peaks at stage 9, could therefore be involved in the activation of these genes. However, from the sequence comparisons of x-rel protein with other members of the rel family (see Section 5.2), it was apparent that x-rel was most likely

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the homologue of the NF-kB constituent, p65, rather than dorsal. NF-kB is constitutively expressed in B cells and is involved in the activation of the immunoglobulin k gene. In addition, NF-kB is involved in the activation of a wide variety of lymphoid-derived factors such as interleukins (for example, interleukin 2 (IL-2) which is produced from T cells; Lenardo and Baltimore, 1989). The known target genes of NF-kB in mammals are likely to be expressed later in development, after the development of the lymphoid system (about stage 50). However, this is not consistent with the expression pattern of x-rel, which peaks at stage 9, although x-rel message is still present at the latest stage tested (stage 35). It may be that a Xenopus homologue of p65, like certain other immunoglobulin transcription factors, for example Oct-2, has an additional role in early development. Oct-2 is expressed in the developing mouse brain in a pattern which suggests a developmental role before its later expression, which is more restricted to lymphoid tissue (He et al., 1989; Rosenfeld, 1991). Whereas expression of these early developmentally-expressed genes may be controlled by homeodomain-containing proteins in Drosophila, such as the bicoid product, there are no known maternally expressed homeodomain gene products in vertebrates. The possible expression pattern of x-rel will be discussed in relation to the phenotypes observed after injection of x-rel message later (see Chapter 13).

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6.3 Primer extension using X.laevis A⁺ RNA.

In addition to Northern analysis of cellular RNA with x-rel probes, I decided to carry out primer extension analysis. The reasons for this were two-fold. Firstly, to give an indication of how close to full-length the cloned cDNAs were. Northern analysis revealed a single hybridising band of about 2kb, suggesting that the cloned cDNAs, x-rel.1 (1940bp) and x-rel.2 (1696bp) were close to full-length. Secondly, from the similarity in sequence between x-rel.1 and x-rel.2 (see Section 5.1), I made the assumption that x-rel.1 and x-rel.2 were derived from the same gene. X-rel.2 is shorter at the 5' end, presumably as a result of premature termination of cDNA synthesis, and at the 3' end, as a result of the use of an alternate polyadenylation signal. If, in fact, x-rel.1 and x-rel.2 were not derived from the same gene, two primer extension products might be observed. Northern analysis may not be of sufficient resolution to indicate the presence of two transcripts. Two (or more) primer extension products would not prove that x-rel.1 and x-rel.2 were products of different genes, but could indicate alternate splicing of the 5' end of transcripts (for example, as observed with the thyroid receptor family), although there is no evidence for this within the rel family.

Two primers were made: Jpe (the 5' end of which is 72 bases from the start of x-rel.1 and 25 bases from the from the start of x-rel.2) and Ape (the 5' end of which is 147

Results and Discussion

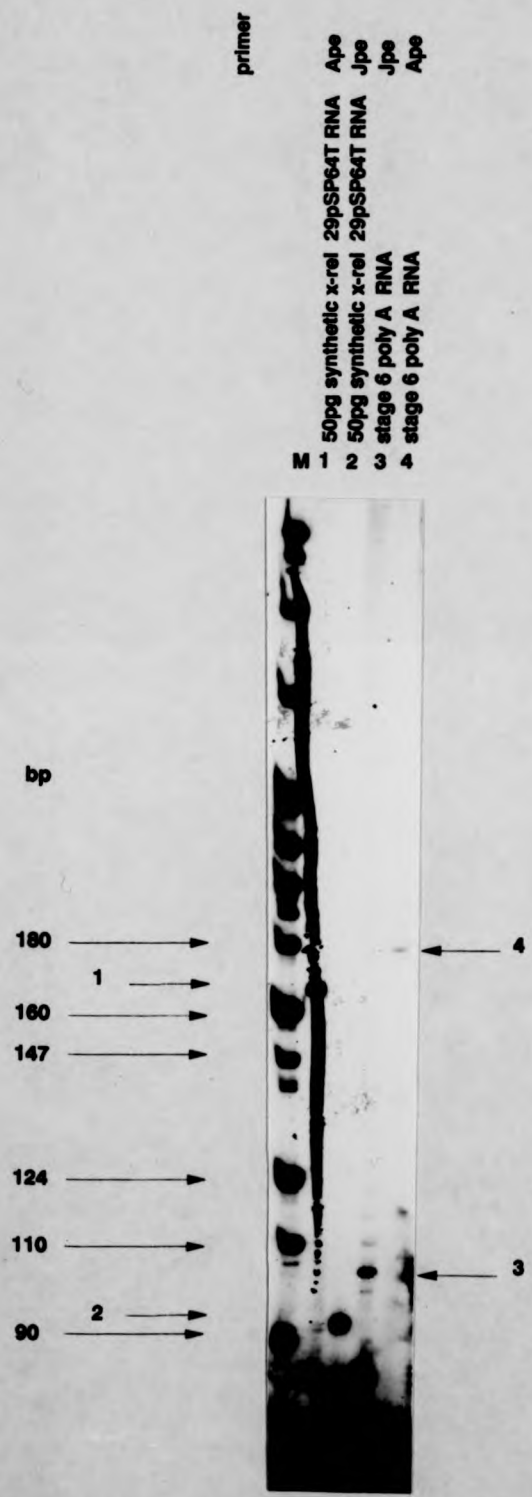
bases from the start of x-rel.1 and 100 bases from the start of x-rel.2). The sequence of these primers is shown in the Appendix. Two primers were made in order to give confidence that the signal observed was from x-rel message - the absolute length of the primer extension products cannot be predicted, but the expected difference in length between observed products is known (i.e. 75 bases). As a control, primer extension analysis of synthetic RNA was carried out. Transcripts were made from x-rel Δ 29pSP64T, which lacked 29 amino acids from its C-terminal end (see Section 7.2). These transcripts contained 69 bases of vector sequences, and consequently the predicted primer extension products are 94 bases for the Jpe primer and 169 bases for the Ape primer.

The results of the primer extension are shown in figure 17. End-labelled primer was hybridised to message overnight, and primers extended by reverse transcriptase. The products were resolved on a 6% denaturing polyacrylamide gel and visualised by autoradiography. The result of primer extension analysis of synthetic transcripts from the x-rel Δ 29pSP64T clone is shown in the tracks indicated. It gave products of the predicted size (94 bases for the Jpe primer and 169 bases for the Ape primer). Primer extension analysis was carried out on poly A⁺ RNA from stage 6. The result is shown in the tracks indicated. The single product from the Ape primer is 178 bases and from the Jpe primer is 103 bases, determined by

Figure 17.

Detection of x-rel transcripts in X. laevis stage 6 embryos by primer extension analysis.

Total RNA was extracted from X. laevis stage 6 embryos and poly A⁺ RNA selected by one round of oligo dT cellulose chromatography. 10ug of poly A⁺ stage 6 RNA was used in each hybridisation reaction; reactions were set up overnight at 45°C. 50pg of synthetic x-relΔ29pSP64T transcript (see Chapter 7) was used as a positive control and to determine how short of full-length x-rel.1 and x-rel.2 were. Two primers, Jpe and Ape (see Appendix), were used to confirm that any x-rel message was genuine. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel. An autoradiograph of the gel is shown. Numbered arrows indicate the main primer extension products for the corresponding tracks. Markers are pBR322 digested with Hpa II and labelled by end-filling. Numbered arrows indicate the main primer extension products for the corresponding tracks.



Results and Discussion

The products were resolved by electrophoresis on a 1.5% agarose gel, which was Southern blotted onto a nylon filter (see Methods section 3.8). The filter was probed with a radioactively labelled x-rel probe, prepared by PCR amplification using three sense primers (JPCR, Jhinc2 and primer 1 - see Appendix). Figure 18 shows that there is a single-stranded product of approximately the correct size (473bp), allowing for the fact that the markers are double-stranded lambda DNA and therefore move with half the mobility. The level of this product was detected to the same extent in RNA prepared from dorsal, middle and ventral sections of stage 17 (neurula) embryos. This even distribution does not necessarily indicate that x-rel is not involved in dorsal-ventral pattern formation in the embryo, since a gradient of dorsal protein with a nucleocytoplasmic distribution is involved in establishing dorsoventral polarity in Drosophila, rather than a gradient at the level of dorsal RNA (or overall gradient of dorsal protein). However, in the light of later experiments (see Chapters 11 and 12) it would clearly be important to extend this experiment to determine the anterioposterior distribution of x-rel message at the gastrula stage of embryogenesis.

Figure 18.

Detection of x-rel transcripts in dorsal, middle and ventral regions of a neurula embryo using a modified primer extension analysis.

Total RNA was extracted from whole, and dorsal, middle and ventral dissections of neurula (stage 17) X. laevis embryos. The equivalent amount of RNA to one embryo was used in the hybridisation with the Jhinc1 primer (see Appendix). After a 1 hour hybridisation at 45°C, the primer was extended by AMV reverse transcriptase (9 units) for 30 minutes at 37°C. After ethanol precipitation and redissolving in 20ul of ddH₂O, the products of the extension were resolved by electrophoresis on a 1.5% agarose gel, which was blotted onto nitrocellulose. The blot was hybridised to radioactively labelled x-rel.2 probe prepared by PCR amplification using the sense primers; JPCR, primer 1, and Jhinc2 (see Appendix). Hybridisation was carried out at 37°C in a solution of 6x SSC, 5x Denhardt's, 50% deionised formamide, 0.1% SDS and 100ug/ml tRNA. The filter was washed in 0.1x SSC at 37°C. An autoradiograph after a 2 week exposure is shown.

RNA extracted from stage 17 embryos

W D M V



526bp

(position of double stranded DNA markers)

Chapter 7.

Expression of x-rel synthetic message in a cell-free system
and in micro-injected oocytes and embryos.

Introduction.

In order to ascertain the function of a cloned gene during development transgenic animals may be produced in which the gene product is overexpressed or expressed ectopically in vivo. Examples of this procedure include studying the functions of oncogenes, growth factors and homeobox genes in mice (Stewart et al., 1984; Thompson et al., 1987; Lang et al., 1987). However, in Xenopus, it is difficult to produce a transgenic organism due to the low frequency of DNA integration and the mosaic expression of introduced genes (Etkin and Pearman, 1987). This occurs in mice but stable lines of transgenic animals are generated, something which the long life cycle of Xenopus laevis does not encourage. Instead, transient expression of gene products has been applied to developing Xenopus embryos using the injection of synthetic mRNAs, for example TFIIIA, Xhox-1A and Xhox3 (Andrews and Brown, 1987; Harvey and Melton, 1988; Ruiz i Altaba and Melton, 1989c). In order to gain an insight into the possible function(s) of x-rel during early embryogenesis we studied the transient overexpression of wild-type and mutated x-rel proteins from micro-injected synthetic transcripts. To do this it was necessary to develop an efficient system for the expression

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of such proteins, and also to determine the expression pattern of proteins translated from injected message. This work is described in this and the following two chapters. Phenotypes of embryos transiently overexpressing x-rel proteins will be described in later sections.

7.1 Expression of x-rel message made from x-rel cDNA in pBluescript.

To determine the size of the x-rel product and to see if it agreed with the size predicted from the open reading frame, capped synthetic sense transcripts were translated in vitro, and in vivo by injection into oocytes. It was important to 'cap' synthetic message (that is, to add a methylated guanosine residue to the 5' end of the message) that was to be injected into oocytes and embryos since it has been shown that the 5' cap structure, found on mature cytoplasmic mRNAs, is essential for their stability in injected oocytes (Lockhard and Lane, 1978). Furthermore, the 5' cap structure is required for the stability of synthetic transcripts injected into oocyte nuclei (Green et al., 1983). Uncapped synthetic message would direct less protein synthesis because of reduced message stability; in oocytes, uncapped RNA has been found to be degraded within 15 minutes, whereas capped synthetic mRNA may be stable for at least 2 days, provided that it has a 3' poly A⁺ tail.

The x-rel cDNA in the vector pBluescript SK⁻ (Stratagene) was linearised with Hind III, and transcribed with T3 RNA

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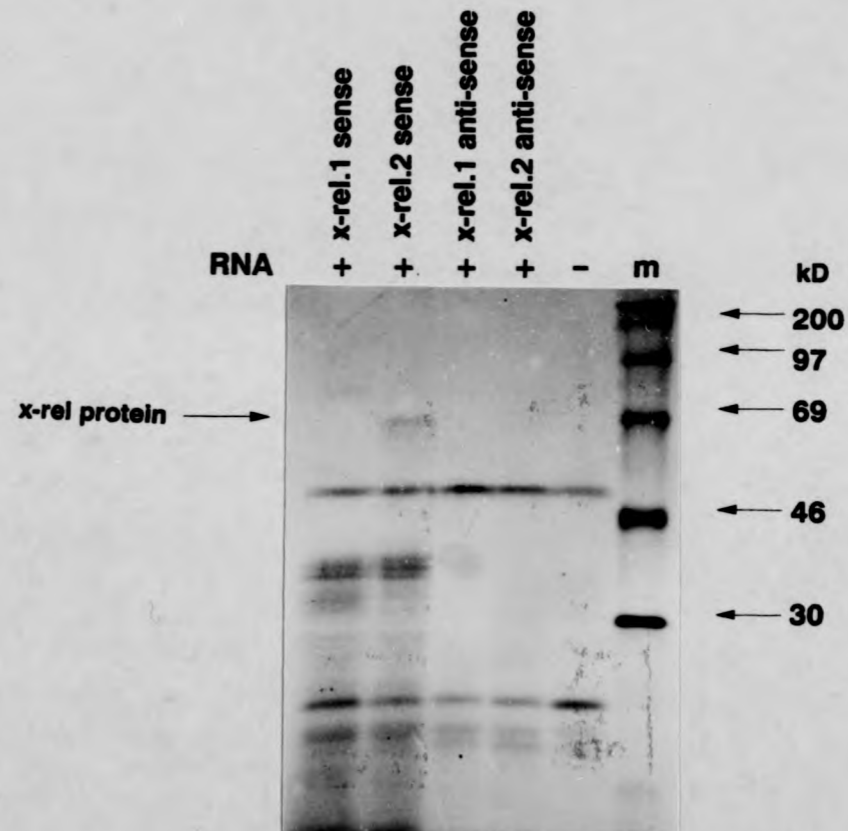
polymerase to produce sense transcripts. Anti-sense transcripts were produced, for use as a control, by linearising with Xba I and transcribing with T7 RNA polymerase. For each in vitro translation, 8ul of rabbit reticulocyte lysate (Amersham) was combined with 1ul of synthetic RNA (about 500 ng/ul) and 1ul (15uCi) of ³⁵S-methionine. After a 45 minute incubation at 30°C, the labelled products were resolved by SDS-PAGE and detected by fluorography. The result is shown in figure 19. As predicted from sequence, x-rel.1, which contains a frameshift mutation, does not produce a full-length product, whereas x-rel.2 produces a product of about 60 kd; there are no extra bands from protein extract produced from anti-sense RNA relative to the control sample containing no added synthetic RNA. The extra base in x-rel.1, compared to x-rel.2, should result in a shortened protein product. This product is predicted to be 280 amino acids long, equivalent to a protein of approximately 30kd. An additional band of this size would not easily be distinguished from background bands seen with x-rel.1 and x-rel.2 translations, and this may be why this band is not visible. The size of the product of x-rel.2 is about 60kd, which is in agreement with the predicted size of 58.5kd from sequence data.

About 20nl of capped sense x-rel.2 transcript (about 250ng/ul) was micro-injected into the cytoplasm of X. laevis oocytes. After an overnight incubation in 5ul/oocyte of Barth X containing ³⁵S-methionine (1.6uCi per oocyte),

Figure 19.

In vitro translation of synthetic transcripts produced from x-rel.1 and x-rel.2.

X-rel.1 and x-rel.2 were linearised with Hind III and Xba I in order to produce templates for sense and anti-sense transcripts respectively. Synthetic transcripts were produced from the pBluescript vectors by using T3 RNA polymerase for sense transcripts and T7 RNA polymerase for anti-sense transcripts. About 500ng of synthetic RNA was mixed with 1 μ l (15 μ Ci) of 35 S-methionine and 8 μ l of rabbit reticulocyte lysate (Amersham). After a 45 minute incubation at 30°C, the labelled products were resolved by SDS-PAGE on a 10% polyacrylamide gel and detected by fluorography. The size and position of radioactive protein molecular weight markers (CFA 626, Amersham) is shown.



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the nuclei were dissected out and nuclear and cytoplasmic labelled products were resolved by SDS-PAGE and detected by fluorography. Figure 23a (shown later) shows that there was no detectable translation of capped synthetic x-rel.2 message, since no extra bands were observed for this sample compared to uninjected oocytes.

7.2 Cloning of x-rel sequence into a high expression vector, pSP64T.

One reason for not being able to detect the expression of x-rel message in oocytes could be that the transcripts produced from x-rel in the pBluescript vector were not very stable. Since continued expression of x-rel message in oocytes and more particularly embryos was of great importance in characterising the x-rel gene, steps were taken to increase the stability of the message in order to be able to enhance expression. A stable synthetic message might allow a sufficient amount of protein to be produced to cause an altered phenotype of injected embryos.

The high expression vector, pSP64T, contains the 5' and 3' untranslated regions of the stable β -globin message (Patient *et al.*, 1983; Krieg and Melton, 1984). Cloning of cDNAs, such as human interferon (Krieg and Melton, 1984) and X1Hbox1 (Wright *et al.*, 1989b), into this vector results in the synthesis of substantial amounts of protein, probably as a result of increased stability of the message transcribed from this vector. By cloning into the Bgl II

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site, which separates these untranslated regions, it may be possible to increase the stability of transcripts of a cloned cDNA sequence, which are produced from the SP6 promoter in this vector. This is not a fusion protein vector and protein synthesis is initiated from the natural ATG of the cDNA. From this point on, the genetic manipulations carried out involved only x-rel.2.

Full-length coding sequence of x-rel.2 (i.e. deletion of the 5' and 3' untranslated regions) was prepared for subcloning into pSP64T by PCR amplification of x-rel.2 sequence using primers that incorporated Bam HI and Bgl II restriction sites. Figure 20 shows the position of the primers in relation to the start and stop codons. The pSP64T vector was cut with Bgl II which produces sticky ends compatible with Bam HI, and the Bam HI/Bgl II x-rel.2 fragment was then ligated into the high expression vector. This construct is called x-relpSP64T. This manipulation maintains a favourable context for the initiation of translation according to Kozak's rules (Kozak, 1989). For a favourable context for initiation, the most important residues are a purine three bases upstream of the AUG and a G immediately after the AUG (PuXXAUGG). The purine (usually A) upstream of the AUG is thought to be most important, with other residues having little effect on the efficiency of translation if this residue is present. However, in the absence of the purine the downstream G is essential. This G residue (and the rest of the coding region) is unaltered by

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region) is unaltered by the manipulation. The important purine, in the 5' untranslated region, is lost.

Deletions of x-rel.2 made from the 3' end were also subcloned into the pSP64T vector (figure 21). The reason for engineering the shorter of the 3' end deletions ($\Delta 29$) was based on the findings of other workers that similar deletions of C-terminal sequence from other rel family members enhances their efficiency of translocation into the nucleus (see Introduction; Section 1.3.4). The larger of the deletions ($\Delta 221$) was engineered to produce a protein that was capable of DNA binding and dimerisation, but lacking activation potential. Such a protein may act as a dominant-negative mutant (Herskowitz, 1987). Dominant negative forms of proteins have been shown to inhibit the normal function of the wild-type protein; for example, a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos, perhaps by disrupting the normal FGF signalling pathway (Amaya et al., 1991) and the normal function of the murine transcription activator, mTFE3 is modulated by a naturally occurring dominant negative form of this transcription factor (Roman et al., 1991).

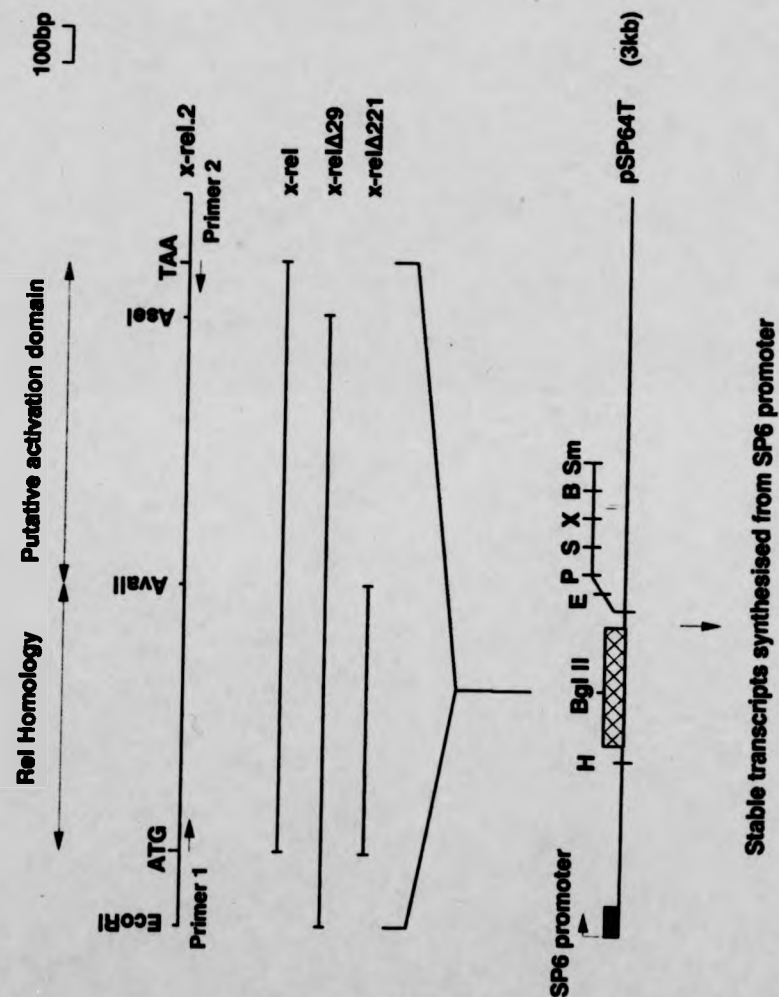
A systematic diagram of the construction of the full-length and deleted x-rel.2 inserts into pSP64T is shown in figure 21. One deletion, x-rel $\Delta 29$, which lacked 29 amino acids from the C-terminus, was made by subcloning a blunted Eco RI-Ase I x-rel fragment, containing some 5'

Figure 21.

Outline of the construction of full-length and deleted x-rel inserts into pSP64T.

X-relpSP64T was constructed by subcloning a PCR amplified product of x-rel coding sequence, using the primers indicated (described in figure 20). The amplified product was digested with Bam HI and Bgl II, sites which are incorporated in the primers, and ligated into the phosphatased Bgl II digested pSP64T vector. X-rel Δ 29 was constructed by subcloning a blunted Eco RI-Ase I x-rel fragment, which contains all of the 47bp of the 5' untranslated sequence and lacks 87bp (29aa) of coding sequence at the C-terminal end, into the blunted phosphatased Bgl II site of pSP64T. X-rel 221 was constructed by subcloning a blunted Bgl II-Ava II fragment derived from the x-rel PCR amplified product (described above) into a blunted phosphatased Bgl II site of pSP64T. The Ava II site occurs just after the end of the N-terminal rel homologous (RH) domain (see Section 1.3.1) and thus lacks the sequence encoding the putative activation domain, which is proline/serine rich (see Section 5.2).

The hatched box represents the β -globin 5' and 3' untranslated regions either side of the Bgl II site in the pSP64T vector (Krieg and Melton, 1984). Linearised DNA was made by digesting x-rel and x-rel 29 with Eco RI and x-rel 221 with Pst I. The filled in box represents the SP6 promoter from which stable transcripts were produced using SP6 RNA polymerase. Restriction sites either side of the β -globin sequence are Hind III (H), Pst I (P), Sac I (S), Xba I (X), Bam HI (B) and Sma I (S).



Results and Discussion

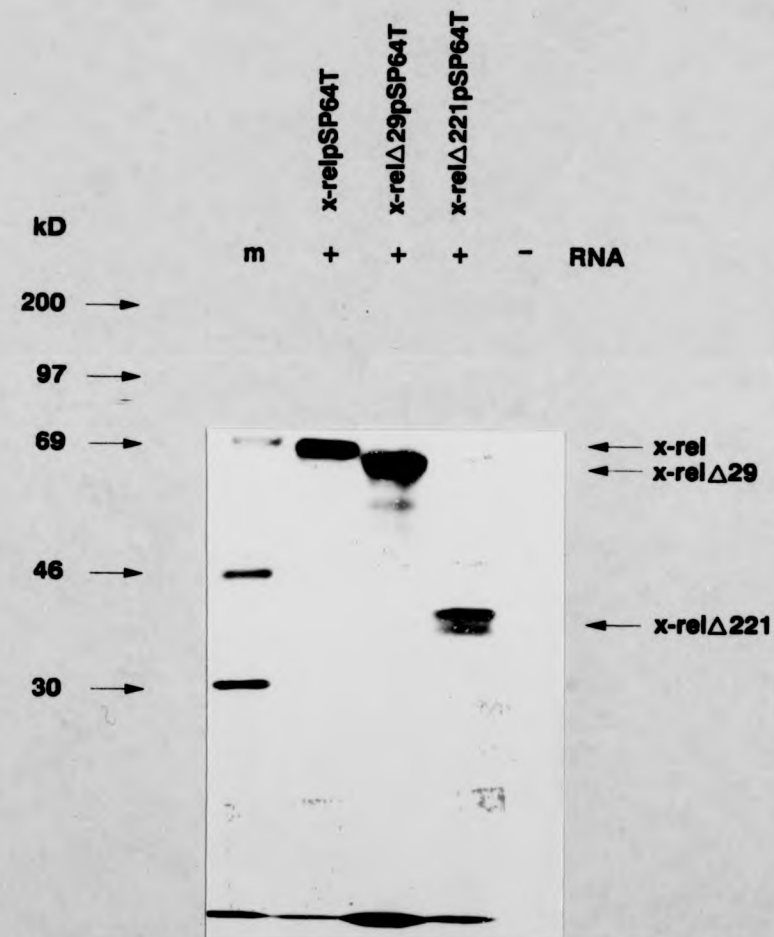
untranslated sequence, into the blunted Bgl II site of pSP64T. The reading frame continues into β -globin 3' untranslated sequence contained in the vector until a stop codon is reached. If the Ase I and vector ends are filled-in by Klenow as expected, (i.e. there is no exonuclease digestion and only polymerisation), then the x-rel coding sequence will be extended by 3 amino acids derived from pSP64T sequence. This is based on the β -globin sequences contained in pSP64T (Krieg and Melton, 1984) and from the sequence of the Bgl II linker used in the preparation of pSP64T, which was not given by Krieg and Melton, (1984), but was determined by sequencing (R. Wales, pers. comm.). This clone is called x-rel Δ 29pSP64T.

The other deletion, x-rel Δ 221, which lacks 221 amino acids from the C-terminus, was made by subcloning a blunted Bam HI-Ava II x-rel fragment, derived from the full-length x-rel PCR product, into the blunted Bgl II site of pSP64T. If the Ava II and vector ends are filled-in by Klenow as expected, then the x-rel coding sequence will be extended by approximately 65 amino acids (the reading frame remains open until the polylinker at which the clone is linearised for transcription) derived from pSP64T sequence. This would result in a translation product of 42kD (35kD of x-rel sequence and about 7kD derived from pSP64T). The observed translation product is slightly smaller than this (about 40kD). However, if the predicted reading frame was not created (for example, as a result of exonuclease activity

Figure 22.

In vitro translation of synthetic transcripts produced from x-rel, x-relΔ29 and x-relΔ221 pSP64T constructs.

X-relpSP64T, x-relΔ29pSP64T and x-relΔ221pSP64T were linearised with Eco R1 and transcribed with SP6 RNA polymerase to produce sense transcripts. 1μl of this RNA was combined with 1μl of ³⁵S-methionine (15μCi) and 8μl of rabbit reticulocyte lysate. The labelled products were resolved by SDS-PAGE on a 10% polyacrylamide gel and detected by fluorography. The size and position of radioactive protein molecular weight markers (CFA 626, Amersham) is shown.



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during end-filling), this would result in the addition of either 3 or 14 amino acids for the other two frames, and such clones would give translation products smaller than that observed. This clone is called x-rel Δ 221pSP64T.

7.3 Expression of synthetic wild-type and deleted message produced from x-rel cDNA in pSP64T in a cell-free system and in oocytes.

X-rel.2 and deletions of x-rel.2 cDNA in the vector pSP64T were linearised with Eco RI and transcribed with SP6 RNA polymerase to produce sense transcripts. 1 μ l of RNA (250ng/ μ l), made from x-relpSP64T, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T, was combined with 1 μ l of 35 S-methionine (15 μ Ci) and 8 μ l of rabbit reticulocyte lysate. After a 45 minute incubation at 30°C, the labelled products were resolved on SDS-PAGE and detected by fluorography. The result is shown in figure 22. Sense transcripts from all three pSP64T constructs, x-rel, x-rel Δ 29 and x-rel Δ 221, express very well and produce easily detected proteins of about the size predicted from sequence data (i.e. 60kd, 58kd and 34kd).

About 20nl of capped 500ug/ml message produced from x-relpSP64T was injected into the cytoplasm of oocytes. After a 4 hour incubation in Barth X containing 35 S-methionine, the nuclei were dissected out of the oocytes and both nuclear and cytoplasmic labelled fractions were homogenised at the end of their labelling time and

Figure 23.

In vivo translation and nuclear targeting of synthetic transcripts produced from x-rel.2 compared to synthetic transcripts produced from x-relpSP64T.

About 20nl of capped transcript (about 250ng/ul) produced from x-rel.2 in pBluescript (a) and from x-relpSP64T (b) was micro-injected into the cytoplasm of X. laevis oocytes. Oocytes were then incubated in Barth X containing ³⁵S-methionine (1.6uCi per oocyte) for 4 hours. The nuclei were dissected out of the oocytes and both nuclear and cytoplasmic labelled fractions were homogenised at the end of their labelling time and resolved on a 10% polyacrylamide gel by SDS-PAGE. The size and position of radioactive protein molecular weight markers, M, (CFA 626, Amersham) is shown.

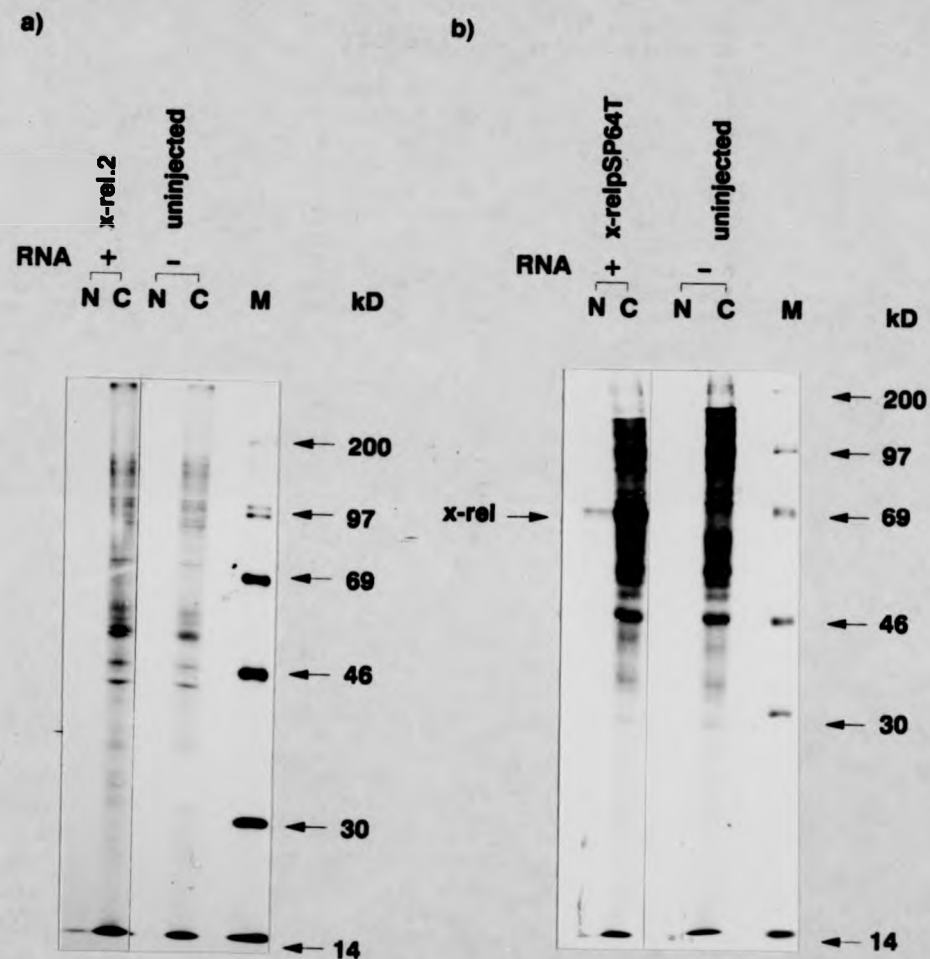


Figure 24.

In vivo translation of synthetic transcripts produced from x-relSP64T, x-relΔ29SP64T and x-relΔ221pSP64T after micro-injection into oocytes and a 4 hour labelling period.

About 10nl of capped transcript (about 250ng/ul) was micro-injected into the cytoplasm of X. laevis oocytes. Oocytes were then incubated in Barth X containing ³⁵S-methionine (1.6uCi per oocyte) for 4 hours. The nuclei were dissected out of the oocytes and both nuclear and cytoplasmic labelled fractions were homogenised at the end of their labelling time and resolved by SDS-PAGE. The size and position of radioactive protein molecular weight markers in the track marked M, (CFA 626, Amersham) is shown.

- a) Short exposure (24 hours) of autoradiograph resulting from labelled proteins produced after a 4 hour incubation of injected oocytes in Barth X containing ³⁵S-methionine.
 b) Long exposure (72 hours) of autoradiograph described in a).

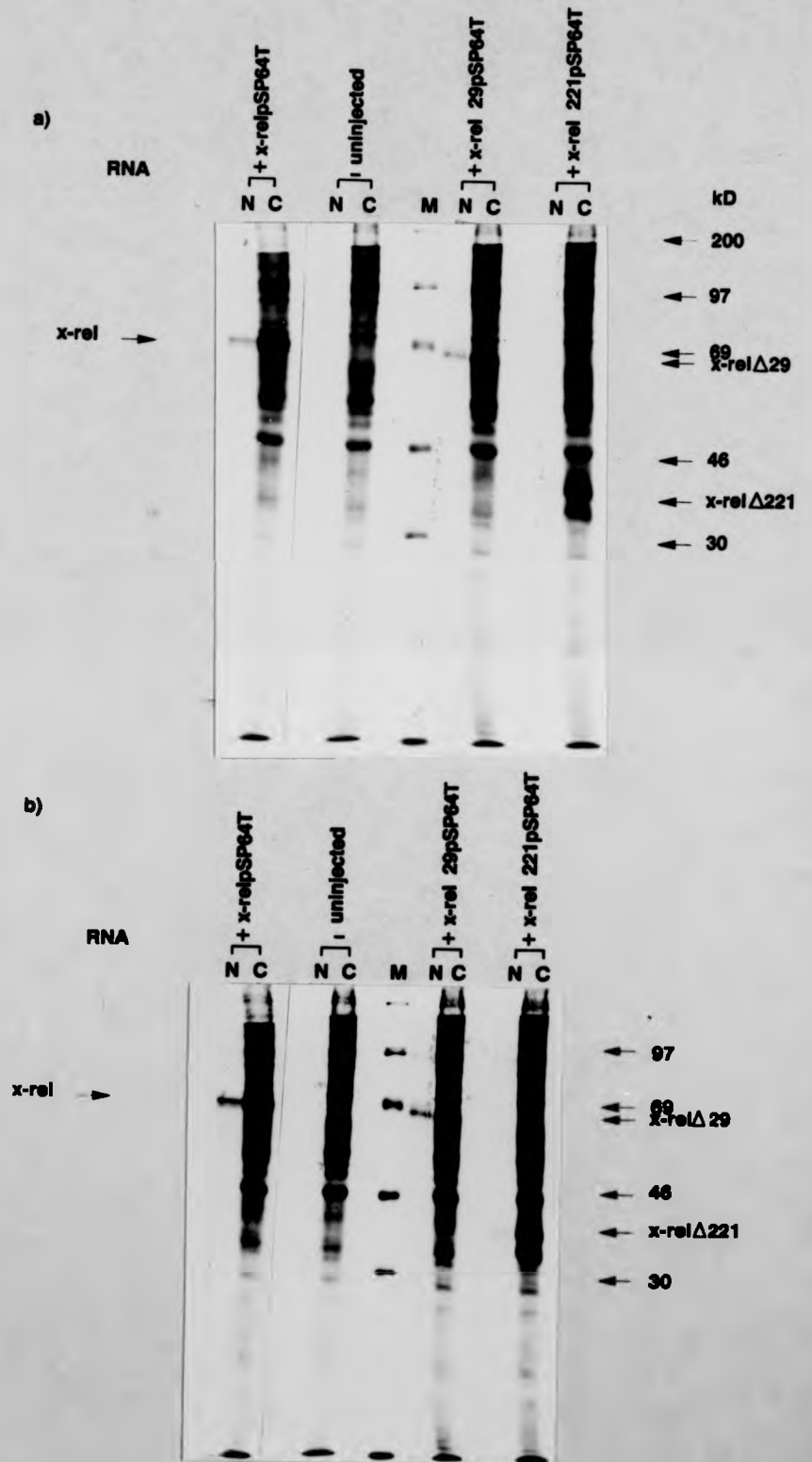
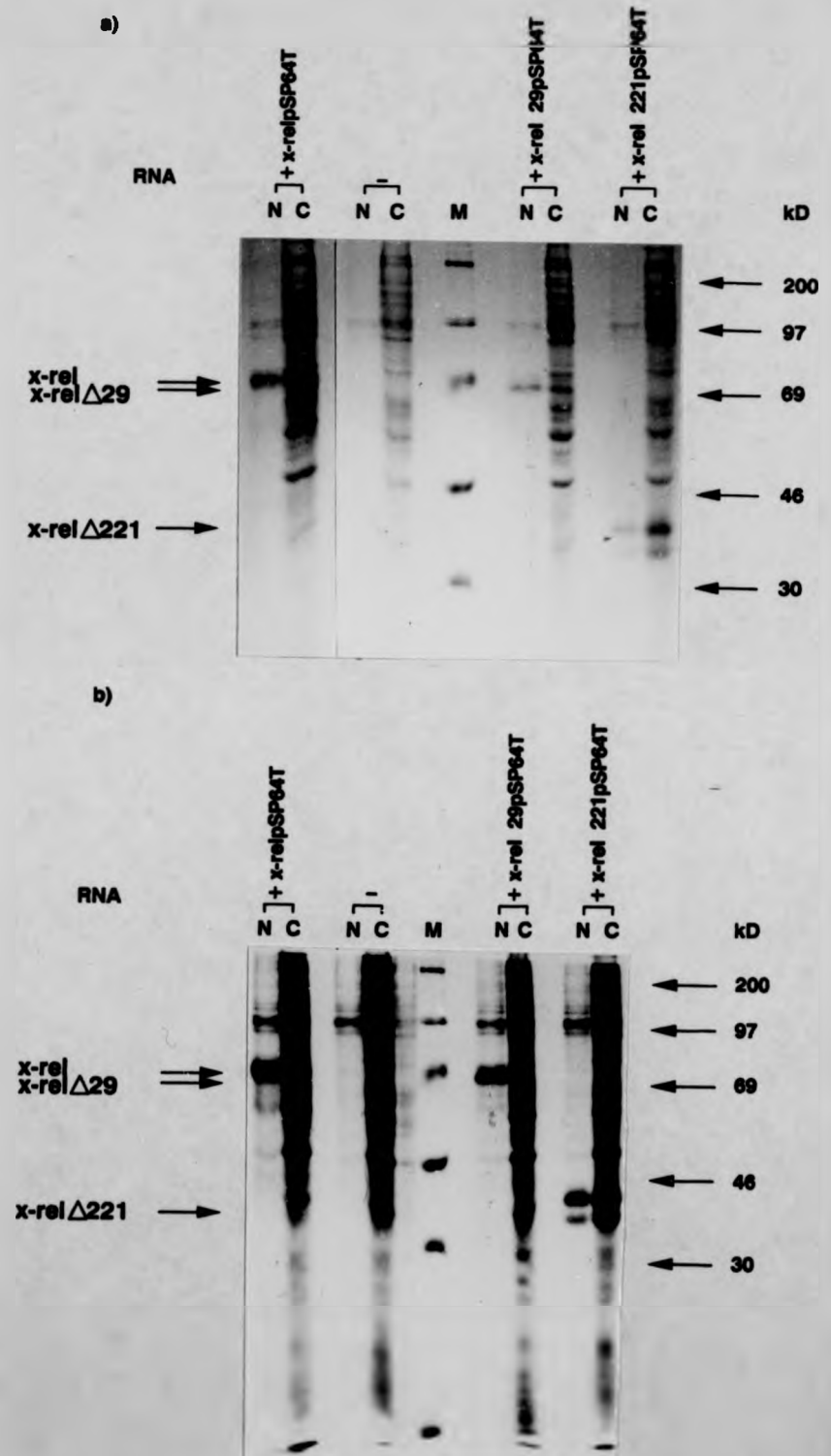


Figure 25.

In vivo translation of synthetic transcripts produced from x-relSP64T, x-relΔ29SP64T and x-relΔ221pSP64T after micro-injection into oocytes and a 24 hour labelling period.

About 10nl of capped transcript (about 250ng/ul) was micro-injected into the cytoplasm of X. laevis oocytes. Oocytes were then incubated in Barth X containing ³⁵S-methionine (1.6uCi per oocyte) for 24 hours. The nuclei were dissected out of the oocytes and both nuclear and cytoplasmic labelled fractions were homogenised at the end of their labelling time and resolved by SDS-PAGE. The size and position of radioactive protein molecular weight markers, marked by M (CFA 626, Amersham), is shown.

- a) Short exposure (12 hours) of autoradiograph resulting from labelled proteins produced after a 4 hour incubation in Barth X containing ³⁵S labelled-methionine.
b) Long exposure (72 hours) of autoradiograph described in a).



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resolved on a 10% polyacrylamide gel by SDS-PAGE. Figure 23 shows the increase in expression of full-length x-rel as a result of cloning into the pSP64T vector (described above) compared with x-rel.2 transcripts produced from the original cDNA clone. There is an extra band clearly visible in both the nuclear and cytoplasmic fractions for the pSP64T clone, but not for the pBluescript clone.

About 20nl of sense transcripts from x-relpSP64T, x-relΔ29pSP64T and x-relΔ22lpSP64T were injected into the cytoplasm of oocytes. To determine whether the labelled proteins produced were stable, oocytes were incubated in Barth X containing ³⁵S-methionine (1.6uCi per oocyte) for 4 and 24 hours. The nuclei were dissected out of the oocytes and both nuclear and cytoplasmic labelled fractions were homogenised at the end of their labelling time and resolved by SDS-PAGE. The resultant fluorographs presented in figure 24 and figure 25 show that transcripts from all three pSP64T transcripts produce an extra band, with respect to the non-injected oocytes, which corresponds to a protein of the predicted size. Furthermore, all three pSP64T transcripts give stable proteins after injection into oocytes, since there is as much labelled protein after 24 hours as there is after 4 hours eventhough all the ³⁵S-methionine is used up after 4-5 hours (see figure 25). Both figure 24 and figure 25 show long and short exposures of these fluorographs to show the band in the nucleus (long exposure) without obscuring the band in the cytoplasmic

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fraction due to the high background of labelled proteins (short exposure). X-rel and x-rel Δ 29 proteins produced from pSP64T transcripts are expressed in a nuclear-cytoplasmic ratio of about 20:80% after 4 hours (figure 24). X-rel Δ 221 protein is only very faintly detected in the nucleus after 4 hours. After 24 hours, proteins from x-rel and x-rel Δ 29 message are expressed in a nuclear:cytoplasmic ratio of 50:50%, but protein from x-rel Δ 221 message is expressed in a nuclear:cytoplasmic ratio of about 30:70%. The apparently lower proportion of protein in the nucleus for the x-rel Δ 221 message could be due to a difference in the kinetics of translocation to the nucleus for this protein. However, it is difficult to compare absolute amounts in the nucleus since x-rel Δ 221 protein is half the size of the other two clones and contains six methionine residues rather than thirteen. Consequently, since a molar equivalent amount of this protein would be half as radioactive, there may be as much of this protein in the nucleus as the other two proteins at each time point. In which case the rate of translocation may be roughly the same for each of the three proteins.

The consensus nuclear localisation signal (NLS) is present in all three clones; the Ava II site occurs just after the NLS and so the shortest deletion, Δ 221, which is truncated at this site, has the NLS. In view of results obtained with C-terminal deletions of other rel family proteins, one might expect x-rel Δ 221 protein to be

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translocated to the nucleus more quickly than x-rel and x-rel Δ 29 proteins. For example, experiments to determine the subcellular location of recombinant v- and c-rel proteins in chicken embryo fibroblasts indicated that the C-terminal half of the c-rel protein determines its cytoplasmic location in CEF and that this sequence can inhibit the v-rel nuclear localising sequence located in the N-terminal half of the v-rel protein (Capobianco *et al.*, 1990; see Introduction section 1.3.4).

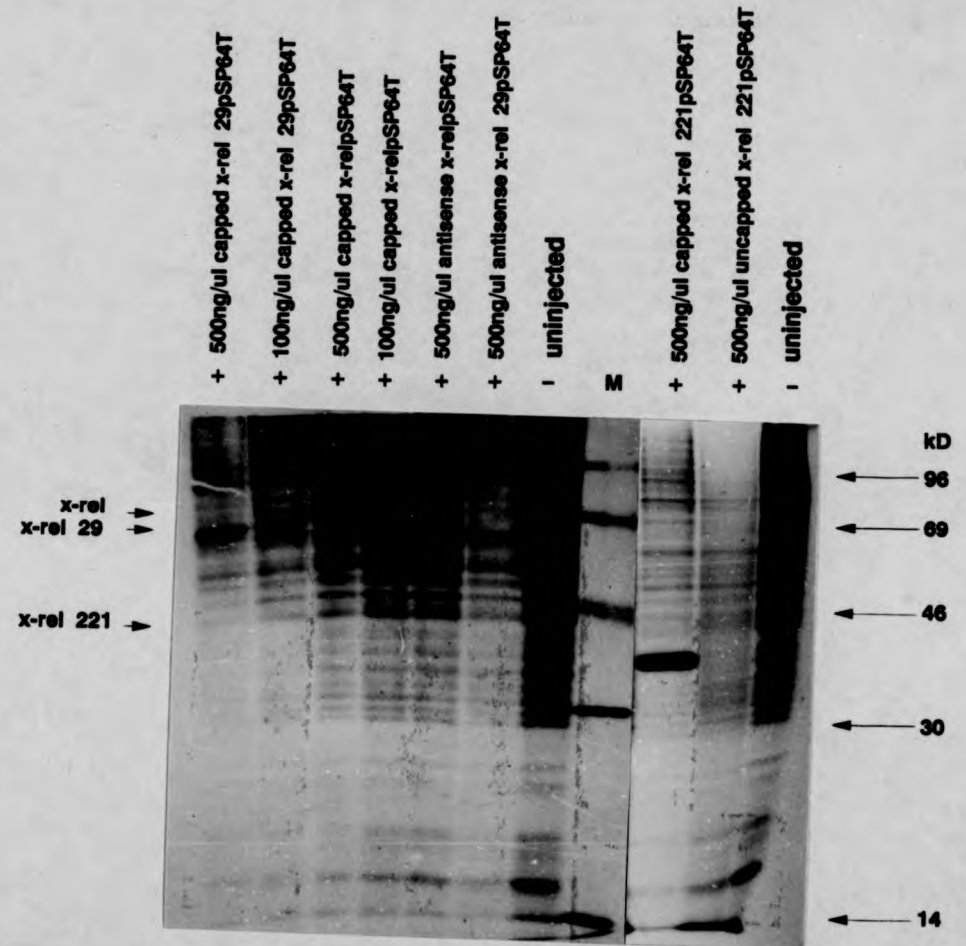
7.4 Expression of wild-type and deleted message from x-rel cDNA in pSP64T in embryos.

The oocyte injection experiments indicated that x-rel synthetic transcripts were efficiently expressed to produce protein products which were stable for at least 24 hours. X-rel transcription is increased in stage 9 embryos, prior to gastrulation, and then declines in late gastrulation (see section 6.2). This may suggest a role for x-rel in embryogenesis at this stage (about 24 hours after fertilisation). Consequently, to investigate the role of x-rel in development by transient overexpression, it would be desirable to ensure expression of x-rel proteins throughout these stages. Given the result of the stability of the proteins, and the observation that β -globin messages are stable in embryos up to stage 8 (Harland and Misher, 1988), it would be expected that these clones would be stable enough for this purpose. The experiment described

Figure 26.

In vivo translation of synthetic transcripts produced from x-relpSP64T, x-relΔ29pSP64T and x-relΔ221pSP64T after micro-injection into embryos.

About 10nl of capped transcripts, of the indicated concentrations, from x-rel, x-relΔ29 and x-relΔ221 pSP64T clones was incorporated by bipolar injections into the cytoplasm of 2-cell stage embryos. Embryos were then injected with 20nl of ³⁵S-methionine that was concentrated to 75uCi/ul and incubated in 1/10x Barth X until they reached early gastrulae, at stage 10. In this particular experiment, capped anti-sense message, prepared at the same time and from the same DNA sample, was injected into embryos as a control for x-rel and x-relΔ29. Uncapped message, prepared at the same time and from the same DNA sample, was injected into embryos as a control for x-relΔ221. Labelled proteins from the homogenised injected embryos were resolved on a 10% polyacrylamide gel by SDS-PAGE. The size and position of radioactive protein molecular weight markers in the track marked, M, (CFA 626, Amersham) are shown. Annotated arrows correspond to proteins of the predicted molecular weight that were produced from the indicated transcripts.



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in this section is designed to ensure that x-rel protein is indeed efficiently expressed from injected transcripts during early embryogenesis.

It was important to 'cap' synthetic message (that is, to add a methylated guanosine residue to the 5' end of the message) that was to be injected into embryos since it is otherwise susceptible to degradation. Capped synthetic message was produced from x-relpSP64T, x-relΔ29pSP64T and x-relΔ221pSP64T as described above. About 20nl of capped transcripts from the three pSP64T clones was injected into the cytoplasm of both cells at the 2-cell stage of development; the concentrations of these transcripts were 500ng/ul or 100ng/ul. Embryos were then injected with 20nl of ³⁵S-methionine that was concentrated to 75uCi/ul, and incubated in 1/10x Barth X until they reached early gastrulae (stage 10). Uncapped synthetic (for x-relΔ221pSP64T) or capped anti-sense message (for x-relpSP64T and x-relΔ29pSP64T), prepared at the same time and from the same DNA sample, was also injected into embryos as a control. Although not shown for all three constructs in figure 26, uncapped message produced from these clones did not produce a translation product, which validated its use as a control for the phenotype experiments described later. Labelled proteins from the homogenised injected embryos were resolved on a 10% polyacrylamide gel by SDS-PAGE and the resultant fluorographs are shown in figure 26. Embryos injected with

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x-relpSP64T, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T capped transcripts all produce an extra band, with respect to embryos injected with uncapped or anti-sense message and non-injected embryos. These bands, which represent strong expression of the relevant gene, correspond to proteins of the molecular weight predicted from sequence.

Chapter 8.

Preparation of x-rel fusion protein constructs.

Introduction.

The reasons for expressing x-rel fusion proteins in E.coli were twofold. The initial reason was to produce large amounts of x-rel protein for use as an antigen in the preparation of polyclonal antiserum. Milligram quantities of x-rel proteins were required for injection of rabbits in order to elicit a strong immune response. A subsequent reason was based on the data emerging during the experiments that most members of the rel family are able to bind to DNA specifically. Production of large amounts of different x-rel fusion proteins may allow the determination of binding activity of the x-rel product in gel electrophoretic mobility shift assays (GEMSA). This chapter specifically describes how the fusion protein constructs were made. The uses of the fusion proteins referred to above are described in detail in later chapters.

The high level expression pET-3 series of vectors (Rosenberg et al., 1987) were used in constructing T7 gene 10 fusions. There are three members (a, b and c) of the pET-3 series, which have a Bam HI cloning site in each of the reading frames. Fusion proteins are produced from clones in the appropriate vector by transforming the constructs into the E. coli strain BL21 (DE3) (Studier and Moffatt, 1986), which has an integrated copy of T7 RNA polymerase under the control of the lac promoter. IPTG

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induces expression of the T7 RNA polymerase from the lac promoter, which transcribes the message for the fusion protein from the T7 gene 10 promoter in pET-3 series vectors.

8.1 Making fusion protein constructs.

X-rel DNA fragments were obtained with Bam HI compatible ends in several ways and subcloned into the Bam HI site of the appropriate pET-3 vector. One construct, pET-3b231, was made by digesting x-rel cDNA with Sau 3A (these sites are shown in figure 10b), which produces Bam HI compatible ends, and subcloning the gel-isolated 0.7 kb fragment (which contains amino acids 176-407) into dephosphorylated pET-3b-Bam HI. This region of x-rel sequence includes at least half of the rel homologous (RH) domain and some of the completely divergent region. Although this fusion protein should have been sufficient to raise polyclonal antiserum, the lack of the complete RH domain, which has been shown to include a DNA binding region in other rel family members, required the construction of another fusion containing all of this region for use in GEMSA studies

A second fusion, pET-3a526, was made by subcloning the full-length x-rel coding sequence into pET-3a. This was carried out by digesting a PCR amplified fragment of x-rel coding sequence, made using the primers (which incorporated Bam HI and Bgl II sites) described in figure 20, with Bam

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HI and Bgl II. The digested x-rel product was ligated into dephosphorylated pET-3a-Bam HI vector.

8.2 Verifying the correct orientation of pET-3b231 and pET-3a526.

Since no common restriction sites were present in the x-rel sequence contained in pET-3b231 to allow verification of the correct 5'-3' orientation, 3 mini-cultures of E. coli BL21 carrying individual recombinants were grown up to determine which one(s) produced an induced band (see Methods Section 3.28.1). A recombinant with the insert in the correct orientation would produce an induced band; a recombinant with the insert in the incorrect orientation would not (there is an in-frame stop codon, TAA, 11 codons after the start of the insert in the other orientation). This construct was transformed into E. coli strain BL21 (DE3) and duplicate cultures for three colonies were grown up in 2x TY medium. When the A₆₀₀ of the cultures reached 0.8, IPTG was added to one of the duplicate cultures for each colony. This induces the expression of T7 RNA polymerase from the lac promoter, which then transcribes message for the T7 gene 10 fusion protein. The other cultures were not induced but were grown for the same period of time; these cultures act as controls since only non-fusion E. coli proteins should be expressed at a detectable level. The proteins produced for both induced and non-induced cultures, were analysed by SDS-PAGE. A

Results and Discussion

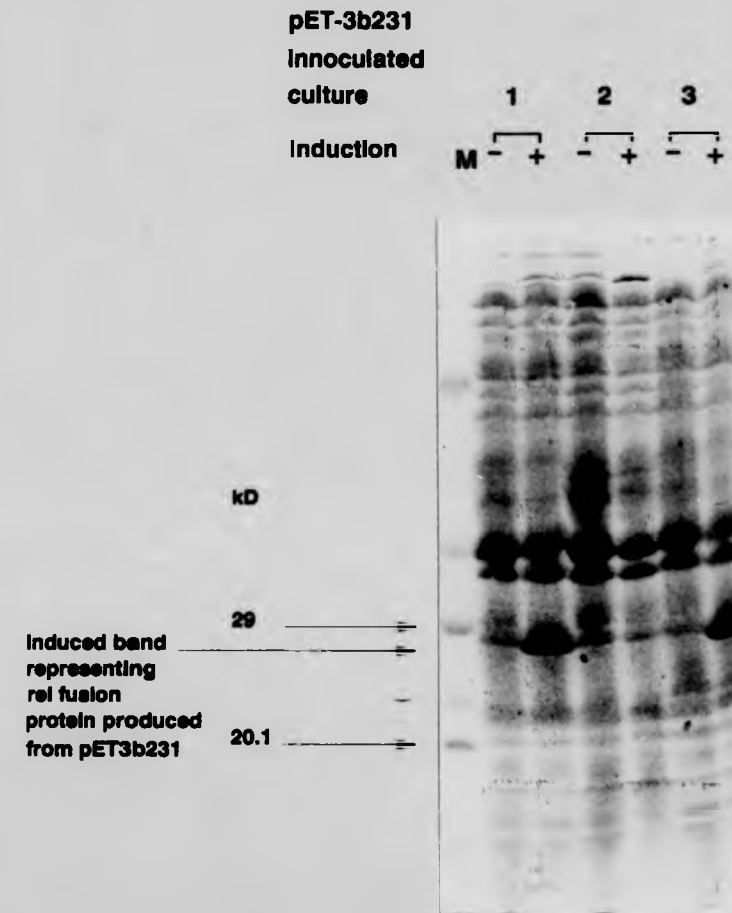
photograph of the acrylamide gel, which was stained with Coomassie blue, is shown in figure 27. An intense band, representing high level expression of the fusion protein, is present for two out of the three induced cultures, with respect to the uninduced cultures. This band represents a protein of about 28kD, which is consistent with the predicted size for pET-3b231 (231 aa of x-rel and 11 aa of T7 gene 10; assuming each amino acid has an average molecular weight of 110) of about 26kD.

To determine whether pET-3a526 contained the coding sequence of x-rel in the correct 5'-3' orientation, the clone was digested with Pst I. As shown in figure 10b, there is a single Pst I site about 280bp from the 3' end of x-rel and a Pst I site in the polylinker of pET-3a which is downstream of x-rel sequence. Since a fragment of approximately 300bp was produced, it was concluded that the x-rel sequence was in the correct orientation with respect to T7 RNA polymerase. This was confirmed by the presence of an intense band, representing expression of the fusion protein, with respect to the uninduced cultures (data not shown). This fusion protein was intended for use in GEMSA (see Section 12.1).

Figure 27.

Coomassie stained acrylamide gel showing the expression of fusion proteins from pET-3b231.

Three cultures, and their duplicates, of BL21(DE3) carrying pET-3b with the 700bp Sau 3A insert were grown to $A_{600nm} = 0.8$, at which point IPTG was added to a final concentration of 1mM to one of each pair of cultures (+). After another 3 hours of growth, the cultures were centrifuged for 5 minutes and resuspended in 100ul of SDS gel loading buffer. 20ul of each sample was resolved by SDS-PAGE on a 12% polyacrylamide gel. The size and position of protein molecular weight markers is shown.



Chapter 9.

Production of an anti x-rel polyclonal antiserum.

Anti x-rel antiserum was required for three main purposes: to detect x-rel protein present in protein extracts on Western blots; to stain x-rel protein present in sectioned oocytes and embryos; and to identify x-rel protein in GEMSA studies.

9.1 Production of x-rel fusion protein for use as an antigen.

The fusion protein construct, pET-3b231, was used as an antigen. As mentioned in Chapter 8, pET-3b321 contains amino acids 176 to 407 of x-rel protein in the vector pET-3b (Rosenberg *et al.*, 1987).

A 100ml culture of BL21 (DE3) carrying pET-3b231 was grown until its absorbance at 550nm (A_{550}) measured 0.8. At this point, the culture was induced by the addition of IPTG (to a final concentration of 1mM). After a further 2-3 hours growth, sonicated protein samples were resolved by SDS-PAGE on a 2x normal thickness 15% polyacrylamide gel. The polyacrylamide gel was lightly stained with Coomassie blue in water (rather than fixer, which would chemically modify the protein and hinder the production of an antibody with affinity for the unmodified, natural protein) to locate the position of the induced band. A gel slice containing the fusion protein was excised and purified by electroeluting the antigen from the acrylamide gel slice

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(see Methods Section 3.28.1). A small fraction of the purified culture was run alongside a pre-determined amount of marker protein to estimate the concentration of the protein solution. The purified protein, resuspended in 1ml of PBS, was used as the antigen in the production of antiserum.

9.2 Production of a polyclonal antiserum.

Aliquots of pET-3b231 fusion protein (in PBS) were freshly emulsified with Freund's complete adjuvant, as described in the Methods section (3.28.2) just before New Zealand White rabbits were given a first injection of 50-100 ug of the emulsified protein. A further three booster injections of the protein emulsified with Freund's incomplete adjuvant, were given at 10 day-2 week intervals. About 5-10 mls of pre-immune serum were taken from the rabbit before the first injection for use as a negative control in subsequent experiments. About 100 mls of immune serum was taken about 10 days after the final injection for use as the neat polyclonal antiserum raised against x-rel protein (Harlow and Lane, 1988).

9.3 Verification that the polyclonal antiserum raised against x-rel reacted with x-rel protein.

Two independent methods were used to verify that the polyclonal antiserum produced reacted with x-rel protein. Firstly, antiserum was tested by using it to stain a

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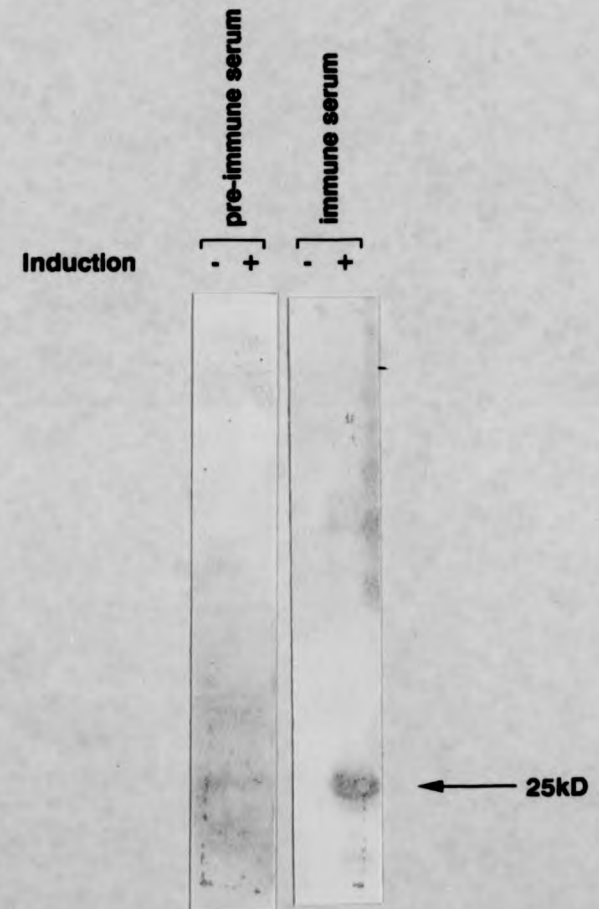
Western blot of protein extract from induced and uninduced E. coli BL21 (DE3) carrying the pET-3b231 fusion protein extract. Proteins were resolved by SDS-PAGE on a 10% separating gel and electrophoretically transferred to a nitrocellulose filter. The filter was incubated in a solution of diluted antiserum, and any antibodies that bound were detected by incubating with a biotinylated secondary antibody, followed by a streptavidin alkaline phosphatase conjugate, and then colour development with BCIP and NBT. The latter two reagents leave a purple-coloured deposit on the filter in a reaction catalysed by alkaline phosphatase. Figure 28 shows a photograph of the stained filter. The antiserum detects a band of the correct size (the fusion protein produced from pET-3b231 is about 25kD) in induced, but not non-induced culture extracts. Pre-immune serum, however, does not detect this band. This method verified that the antiserum reacts with x-rel protein produced in E. coli.

The second method of immunoprecipitation was used to determine whether the polyclonal antiserum reacted with x-rel protein produced in oocytes (which had been incubated in ³⁵S-methionine) injected with x-rel synthetic message. Duplicate samples from oocytes injected with synthetic message from x-rel.2, x-relΔ29pSP64T, and from oocytes that had not been injected, were prepared for immunoprecipitation using S. aureus protein A (see Methods section 3.31). The equivalent of one oocyte was used for

Figure 28.

Western blot showing that the polyclonal antiserum raised against x-rel reacted with x-rel pET-3b231 fusion protein.

Duplicate samples of protein extract from 100ul of induced (+ tracks) or non-induced (- tracks) cultures of E. coli transformed with pET-3b231 was resolved by SDS-PAGE on a 10% polyacrylamide gel, and electrophoretically transferred to a nitrocellulose filter. One half of the filter was incubated in a 1:100 dilution of pre-immune serum. Bound antibodies were detected as described in Method section 3.28. Antiserum, but not pre-immune serum, detected the pET-3b231 fusion protein (about 25kD) in the induced (but not non-induced) extract.



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each duplicate sample and 2ul of pre-immune and immune serum were added to respective samples. The precipitated samples were analysed by SDS-PAGE and the resultant fluorograph is shown in figure 29. A labelled protein band (of the correct size) was immunoprecipitated by antiserum only from extract from oocytes that had been injected with x-rel Δ 29pSP64T message. No x-rel protein was precipitated from the sample containing oocytes that had been injected with message from x-rel.2; this was not surprising as it was previously shown (Section 7.1) not to produce quantities detectable by analysis of labelled proteins by SDS-PAGE. No precipitated band was observed using the pre-immune serum.

The solitary band, referred to in figure 29 is an indication of the specificity of the antiserum. X-rel protein could not be precipitated from samples containing oocyte extract that had not been injected with synthetic message and therefore this method was not sensitive enough to detect endogenous x-rel protein, if it was indeed present. The fact that there is x-rel message in oocytes (refer to Chapter 4) does not necessarily indicate that there is x-rel protein present; if x-rel protein is present it would only be labelled if being actively synthesised in oocytes of the stage injected, which may or may not be the case. Some proteins are synthesised early in oogenesis and then synthesis stops.

Antiserum was purified by affinity for the fusion protein

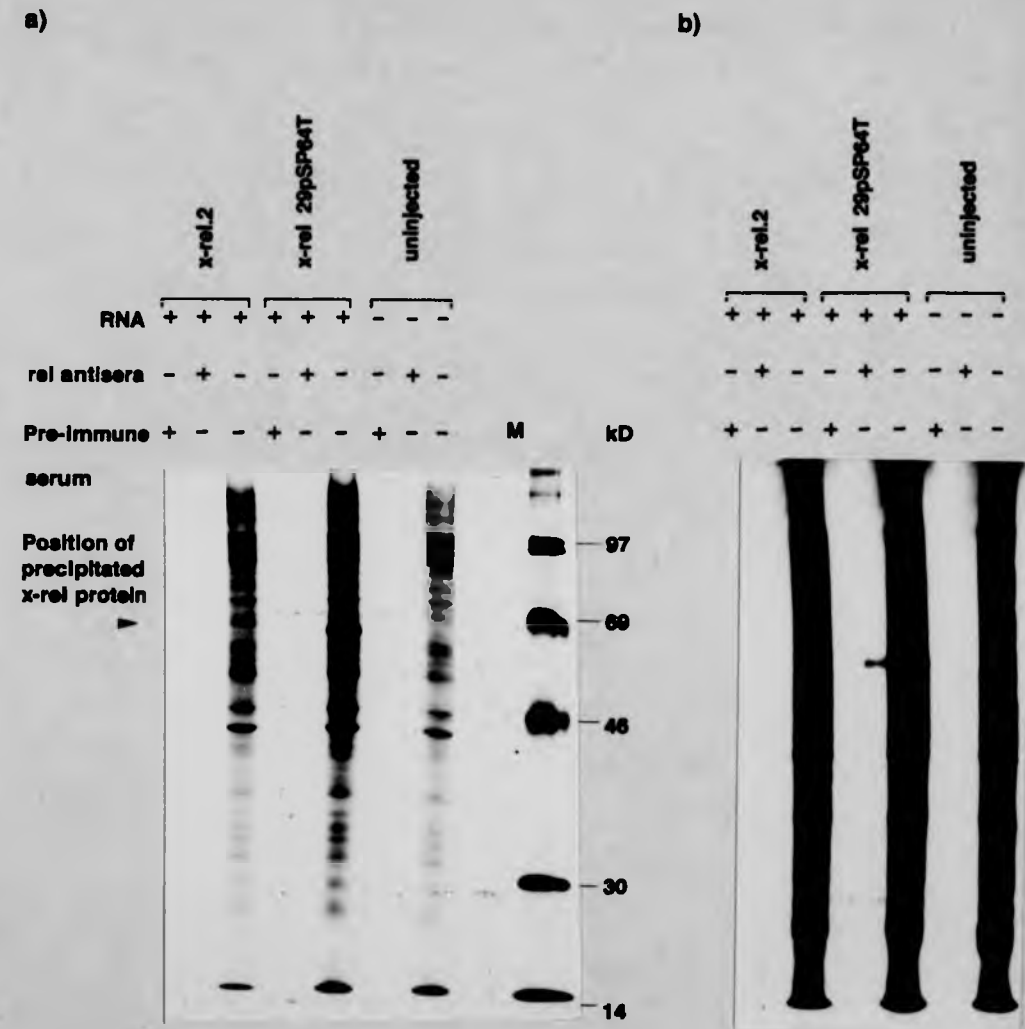
Figure 29.

Immunoprecipitation showing the specific reaction of the polyclonal antiserum raised against pET-3b231 with x-rel protein produced from oocytes injected with x-rel Δ 29pSP64T message.

Duplicate samples from ^{35}S -methionine labelled oocytes that had been injected with 20nl of 250ug/ml of synthetic message from x-rel.2, x-rel Δ 29pSP64T, and from ^{35}S -labelled oocytes that had not been injected, were prepared for immunoprecipitation using S. aureus protein A. The equivalent of one oocyte was used for each duplicate sample and 2ul of pre-immune and immune serum were added to respective samples. The precipitated samples were analysed by SDS-PAGE on a 10% polyacrylamide gel. The band indicated represents a labelled protein band (of the correct size) that was immunoprecipitated by antiserum only from extract from oocytes that had been injected with with x-rel Δ 29pSP64T message. No precipitated band was observed using the pre-immune serum.

a) Autoradiograph after a 12 hour exposure of the labelled proteins produced after immunoprecipitation.

b) Autoradiograph after 1 week exposure of the labelled proteins produced after immunoprecipitation.



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produced from pET-3b231, and electrophoretically transferred to a nitrocellulose filter as described in Methods Section 3.28.3. However, this purification step did not seem to reduce the amount of background when purified and unpurified antiserum were compared in the staining of Western blots of X. laevis protein extract (data not shown), and so was not used for any of the experiments subsequently discussed.

Chapter 10

Detection of x-rel protein in oocytes and embryos using polyclonal antiserum raised against pET-3b231 x-rel protein.

This chapter describes the detection of x-rel protein in oocytes and embryos that had been injected with synthetic x-rel message, with the antiserum raised against pET-3b231. The detection of x-rel protein produced in this way, was conducted either on Western blots of oocyte/embryo extract or by staining oocyte and embryo sections with fluorescently labelled antibody. These methods were also used to try to detect endogenous x-rel protein with the antiserum raised against pET-3b231.

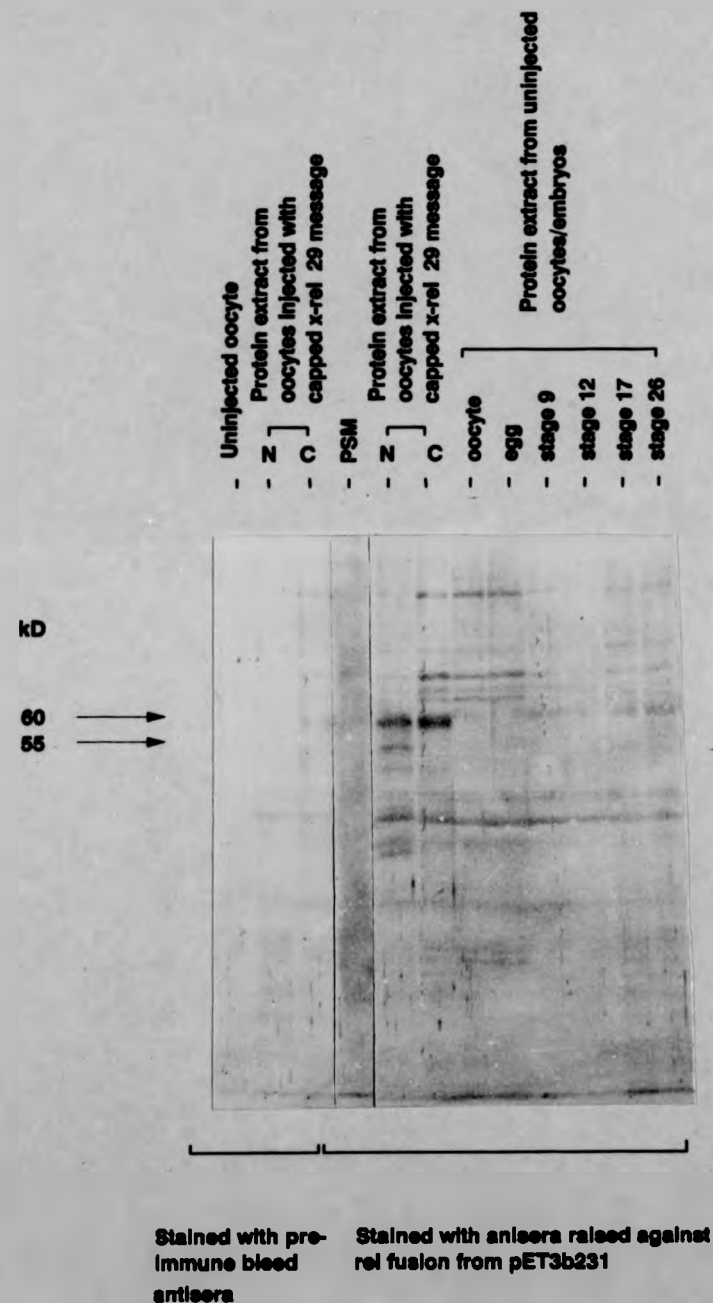
10.1 Detection of x-rel protein using polyclonal antiserum by Western blotting.

X. laevis oocyte and embryo extracts and the nuclear and cytoplasmic fractions of oocytes that had been injected with x-rel $\Delta 29$ message, were resolved by SDS-PAGE on a 10% polyacrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose filter and the antibody that bound, after incubation with polyclonal antiserum, was detected as described in Section 9.3. A photograph of the filter is shown in figure 30. The antiserum detected a band of about 60kD, which corresponds to the predicted size of x-rel protein, in cytoplasmic extract from oocytes that had been injected with x-rel $\Delta 29$ message, relative to those

Figure 30.

Western blot showing strong reaction of polyclonal antiserum raised from pET-3b231 with x-rel protein.

Protein extract was prepared from the nuclear (N) and cytoplasmic (C) fractions of oocytes injected with x-rel Δ 29 message and from uninjected oocytes and embryos of the indicated stage. The equivalent of 1 oocyte/embryo was resolved on a 10% polyacrylamide gel, which was electrophoretically transferred to nitrocellulose. The filter was incubated with polyclonal antiserum and the antibodies that bound were detected as described in section 9.3. The position of the 60kD protein and extra 55kD nuclear protein are indicated, as determined from the position of pre-stained markers (PSM).



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detected by pre-immune serum (the background on the filter stained with antiserum was very high, but the bands described above were above background). These bands were not present for the oocyte samples that had not been injected with x-rel message. In the nuclear fraction, a smaller band of about 55kD was detected in addition to the 60kD band detected with both the nuclear and cytoplasmic fraction. Modification of the x-rel protein could, therefore, be taking place. This could be phosphorylation since there is a consensus site (RRXS) for phosphorylation by protein kinase C at amino acids 272-275 (RRPS); the negative charge would increase the mobility of the protein (however, such a large difference in size suggests that this is not the case). Alternatively, the modification could be due to glycosylations or some form of proteolytic processing. This smaller 55kD band is not detected in the cytoplasmic fraction, but since full-length protein is detected in the nucleus, this processing does not appear to be essential for translocation to the nucleus and probably only occurs in the nucleus. This smaller band was sometimes detected in the nuclear fraction of oocytes used in the labelling experiments described in Chapter 7.

Antiserum detected no additional bands in uninjected oocyte and embryo extract, relative to the blot incubated with pre-immune serum. This method of detecting x-rel protein was therefore not sufficiently sensitive to detect endogenous x-rel protein from X. laevis oocyte and embryo

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extracts. It is possible that there is a low abundance of x-rel protein in the early X. laevis embryo since RNA analysis also showed a low level of x-rel transcripts (section 6.2). Furthermore, the small number of clones with homology to x-rel that were derived from an oocyte library reflected the low abundance of stored x-rel message, and therefore probably protein.

10.2 Detection of x-rel protein by staining X. laevis oocyte and embryo sections with rel antibody and counter-staining with a fluorescently-labelled antibody.

The Western blot data indicated that the antiserum raised against pET-3b231 reacted with x-rel protein. In order to determine the distribution of x-rel protein, sections of oocytes and embryos that had been injected with synthetic x-rel message were made, incubated with rel antibody and then counter-stained with fluorescently labelled secondary antibody.

Since the antibody was raised against a fusion protein that had been purified by SDS-PAGE and might perhaps only react with denatured x-rel protein, a method involving treating sectioned oocytes and embryos with SDS prior to staining with the antibody, was adopted. This method improved the sensitivity of detections over conventional procedures; indeed little reaction was obtained without the SDS treatment (data not shown). Oocytes and embryos were

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fixed and sectioned as described in Methods section 3.29. They were subjected to a final concentration of 0.1% SDS after rehydration with PBS following fixation. After incubation with anti-rel antibody and then FITC conjugated goat anti-rabbit IgG (Sigma) diluted 1:50 in PBS for 30 minutes at room temperature, they were washed in PBS and mounted in PBS/glycerol (1:1). Stained sections were viewed with a Nikon optiphot lens and the images recorded on ASA 160 Ektachrome film. Figure 31 shows a section of an oocyte that had been injected with uncapped x-rel Δ 29pSP64T message for use as a control. After staining with anti-rel antibody and then the FITC-conjugated goat anti-rabbit IgG, this section was viewed under white light (Figure 31a) and under fluorescent light (Figure 31b). Evidently, there is no clear staining by the FITC-labelled secondary antibody indicating that the rel antibody cannot react with any endogenous x-rel protein that may be present. Figure 32 illustrates a section through an oocyte that had been injected with capped x-relpSP64T message. After staining with anti-rel antibody and then the FITC-conjugated goat anti-rabbit IgG, this section was viewed under white light (Figure 32a) and under fluorescent light (Figure 32b). There is clearly nuclear staining by the FITC-labelled secondary antibody for this oocyte demonstrating that there is a high degree of concentration of the protein synthesised from x-rel Δ 29pSP64T message in the nucleus (as was shown by SDS-PAGE for all three x-rel pSP64T messages;

Figure 31.

Section of control oocyte injected with 20nl of 500ug/ml x-rel Δ 29 uncapped message, stained with a 1:50 dilution of rel antibody (antibody raised against rel fusion protein produced from pet3b231) and counter-stained with a 1:50 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma).

a) Viewed under white light.

b) Viewed under fluorescent light.

There is an overall general background staining, but no specific staining of the nucleus.

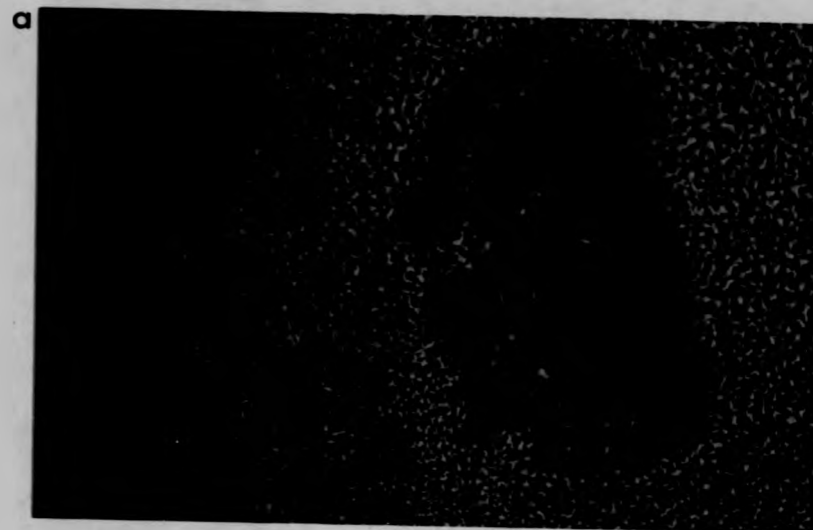


Figure 32.

Section of oocyte injected with 20nl of 500ug/ml x-rel Δ 29 capped message, stained with a 1:50 dilution of rel antibody (antibody raised against rel fusion protein produced from pet3b231) and counter-stained with a 1:50 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma).

a) Viewed under white light.

b) Viewed under fluorescent light.

The nucleus is clearly stained stronger than the cytoplasm. Cytoplasmic staining is stronger than in the control section (figure 31b).



Figure 32.

Section of oocyte injected with 20nl of 500ug/ml x-rel Δ 29 capped message, stained with a 1:50 dilution of rel antibody (antibody raised against rel fusion protein produced from pet3b231) and counter-stained with a 1:50 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma).

a) Viewed under white light.

b) Viewed under fluorescent light.

The nucleus is clearly stained stronger than the cytoplasm. Cytoplasmic staining is stronger than in the control section (figure 31b).



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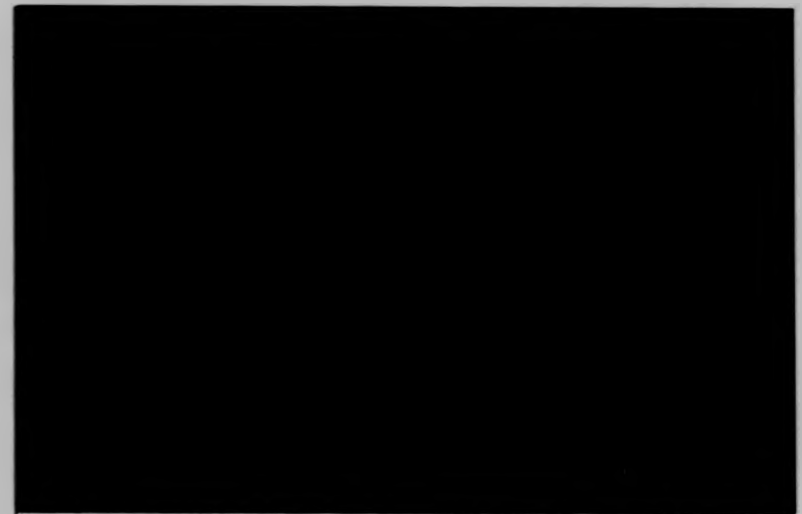
see Section 7.3). A low level of staining, which is higher than the background staining observed in figure 31b) is observed in the cytoplasm of this oocyte, which reflects that translocation of x-rel Δ 29 protein to the nucleus is not total.

Figure 33 shows a section through the dorsal side of a stage 10 embryo that had been injected with x-rel Δ 29pSP64T message at the 2-cell stage. Viewed under fluorescent light this section shows that there is an overall staining by the FITC-labelled secondary antibody which appears more distinct in the nuclei, indicating that x-rel protein is concentrated in the nucleus. In this embryo, no such staining on the ventral side of the embryo was observed (data not shown), suggesting the potentially interesting result that x-rel protein may be localised on the dorsal side of the embryo. However, the result may derive from an uneven distribution of the mRNA. It was not clear that the effect was reproducible, and in general it is not easy to distinguish dorsal and ventral sides of these sections of stage 9 and stage 10 embryos. Evidently, more work is required to clarify this result.

The main conclusions from these stained sections are firstly that the rel antibody, raised against the fusion protein produced from pET-3b231, reacts with x-rel protein that was synthesised as a result of injection of x-rel Δ 29pSP64T message into oocytes and embryos, but unfortunately detection was not sensitive enough to detect

Figure 33.

Section through dorsal side of stage 10 embryo, which had been injected with x-rel Δ 29 capped message at the two-cell stage and stained with a 1:50 dilution of rel antibody (antibody raised against rel fusion protein produced from pet3b231) and counter-stained with a 1:50 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma).



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endogenous x-rel protein. Secondly, nuclear localisation occurs at oocyte and blastula stages.

Chapter 11.

Detection of *X. laevis* proteins that bind to kB-like binding sites.

11.1 Introduction.

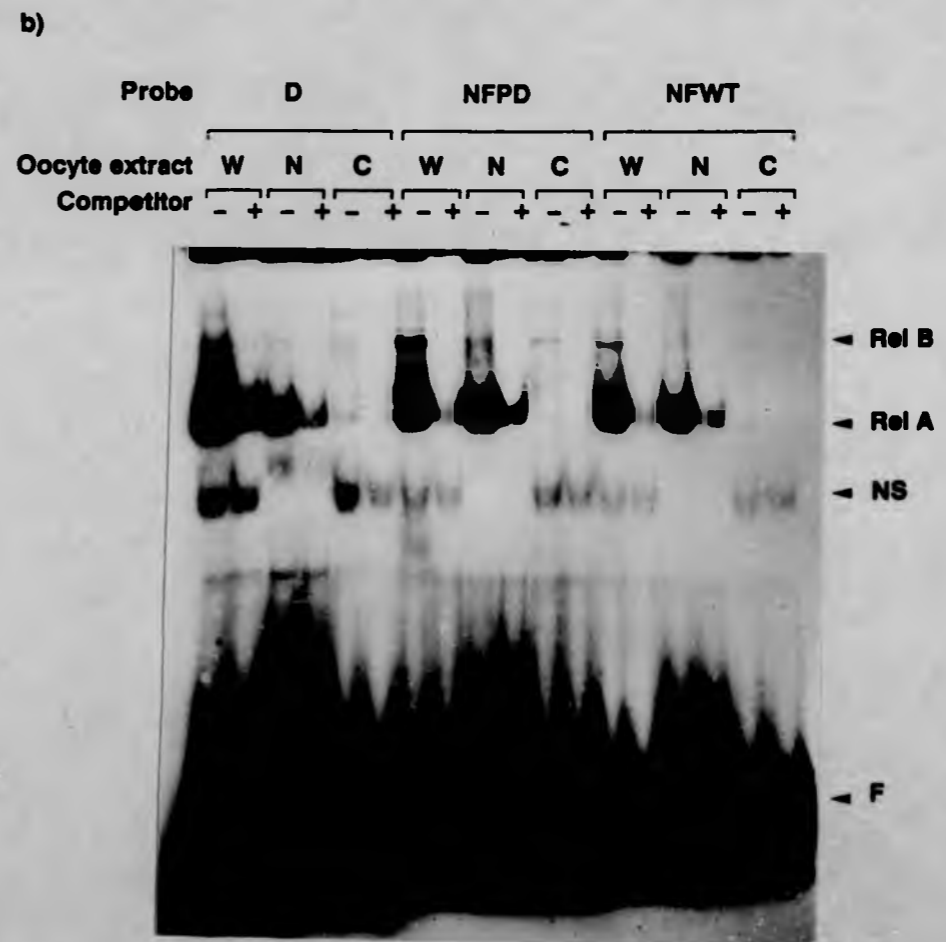
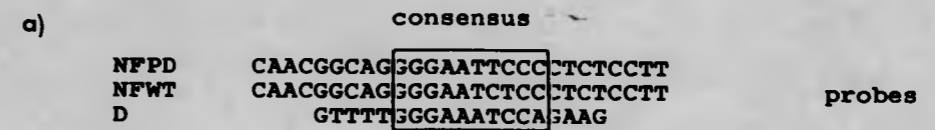
NF-kB and v-rel/c-rel proteins have been shown to bind to kB enhancer sites in vitro (Inoue et al., 1991). Dorsal has also been shown to bind to sequences that resemble NF-kB-like sequences (Ip et al., 1991). In order to determine whether *X. laevis* proteins exist that can bind to kB-like sequences, band shift assays were carried out using three double-stranded oligonucleotide probes; NFPD, NFWT and D (see figure 34). The sequence of these probes was based on published data, referred to above, using NF-kB (immunoglobulin k chain enhancer) and dorsal (from the zen promoter) binding sites. Each probe contained a consensus decamer sequence, shown in figure 34, surrounded by flanking sequence. NFPD and NFWT contained identical flanking sequences and differed by only 2 base pairs in their consensus sequence to give a perfectly palindromic and a wild-type sequence respectively (see Introduction Section 1.3.3). Gel shift assays by other workers have indicated that (p50)₂ preferred the palindromic site, p(65)₂ bound equally to both sites and p50-p65 (NF-kB) preferred the wild-type binding site (see Introduction section 1.3.3). Dorsal protein is able to bind to the D sequence motif which was identified in the zen promoter, and which is similar to the consensus binding site

Figure 34.

a) Sequence of the upper strand (5'-3') of probes containing kB enhancer binding sites which were used in band shift assays to show that oocyte extract contains proteins that bind to the kB enhancer site.

b) Gel shift assay showing binding of proteins in X. laevis oocyte extract to kB enhancer binding sites.

Oocyte extract was prepared from oocytes of X. laevis (see Methods section) and the equivalent of one half of an oocyte was used in each binding reaction. W indicates whole oocyte extract, C indicates cytoplasmic (enucleated oocyte) extract and N indicates nuclear extract. The three probes used all contained a consensus kB binding site (see above). An excess of unlabelled probe was added to every other binding reaction as competitor (marked by +). The positions of Rel A, Rel B, non-specific complexes (NS) and free probe (F) are indicated.



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described for p50-p65 (Ip et al., 1991). Both of these sequences are similar to the dorsal-binding motif identified in the twist promoter (Thisse et al., 1991), but the symmetry observed for zen and wild-type kB binding sites is much lower than for the twist dorsal-binding sites. Furthermore, the affinity of dorsal for the zen motif is about five times more than for the twist motif.

11.2 Two kB enhancer binding proteins can be detected in X. laevis oocyte extract.

X. laevis band shift extract was prepared as described in Methods Section 3.33.1. The equivalent of one half of an oocyte of extract was normally used in these assays. Extract was made from whole oocytes, enucleated oocytes (cytoplasmic fraction) and nuclei. The three probes referred to in figure 34a were used in these assays. The result of these band shift assays using oocyte extract is shown in the autoradiograph presented in figure 34. Specific binding can be competed out by the addition of an 80-fold excess of unlabelled probe in the binding reaction; non-specific binding cannot. Two bands representing specific kB-binding proteins were detected in X. laevis oocyte extract. One of these bands, referred to as Rel A (of the greater mobility), represented a binding protein that was detected for all three probes. The other band, Rel B, represented a binding protein whose activity was only strongly detected with the NFPD and NFWT probes; a weak and

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band in this position was usually observed with the D probe. Although referred to as Rel A and Rel B, there was no evidence that these proteins were related to rel family proteins, but they shared the property of being able to bind to kB-like sequences. Other bands of lower mobility were observed, but these were not consistently observed and not always efficiently competed.

11.3 Location of Rel A and Rel B in the X. laevis oocyte.

The band shift assay referred to above and shown in figure 34 indicated that Rel A and Rel B DNA binding activities are unequally distributed between the nuclear and cytoplasmic fractions of the oocyte. Approximately 95% of Rel A binding activity detected in the oocyte was present in the nuclear fraction. Rel B binding activity was detected at a much lower and more inconsistent level in both the nuclear and cytoplasmic fractions, compared to whole oocytes, conceivably due to the dissection and homogenisation treatment being too harsh for this protein, which may be less stable than Rel A. Dissection of embryos was also shown to result in the loss of Rel B binding activity (see section 11.5). However, Rel B binding activity is detected slightly more in the cytoplasm than in the nucleus.

NF-kB (p50-p65) is not detected in the cytoplasm of mammalian cells (Baeuerle and Baltimore, 1988). The low level of p50-p65 binding activity in the cytoplasmic

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fraction might be due either to the association of the cytoplasmic inhibitor protein, I κ B, with p65 or to its active concentration in the nucleus (see Introduction section 1.3.4). I κ B probably masks the DNA binding site of p50 and p65 and it might mask the nuclear localisation signal of p65, thereby preventing nuclear entry of the p50-p65 dimer. Alternatively, I κ B might anchor the complex to the nucleus by association with the cytoskeleton; MAD-3, thought to encode I κ B, has ankyrin repeats suggesting a role in attachment to the cytoskeleton (see Introduction section 1.3.4). Rel A may either be anchored in the cytoplasm by an inhibitor protein or may not exist in the cytoplasm at all. However, detergent treatment of gel shift extract (described later; data not shown) did not increase the binding activity of Rel A in the cytoplasm (or whole extract). Some Rel B binding activity, on the other hand, is detected in the cytoplasm without requiring detergent treatment of the band shift extract and is in this respect dissimilar to p50-p65.

11.4 Temporal Distribution of Rel A and Rel B in early X. laevis embryogenesis.

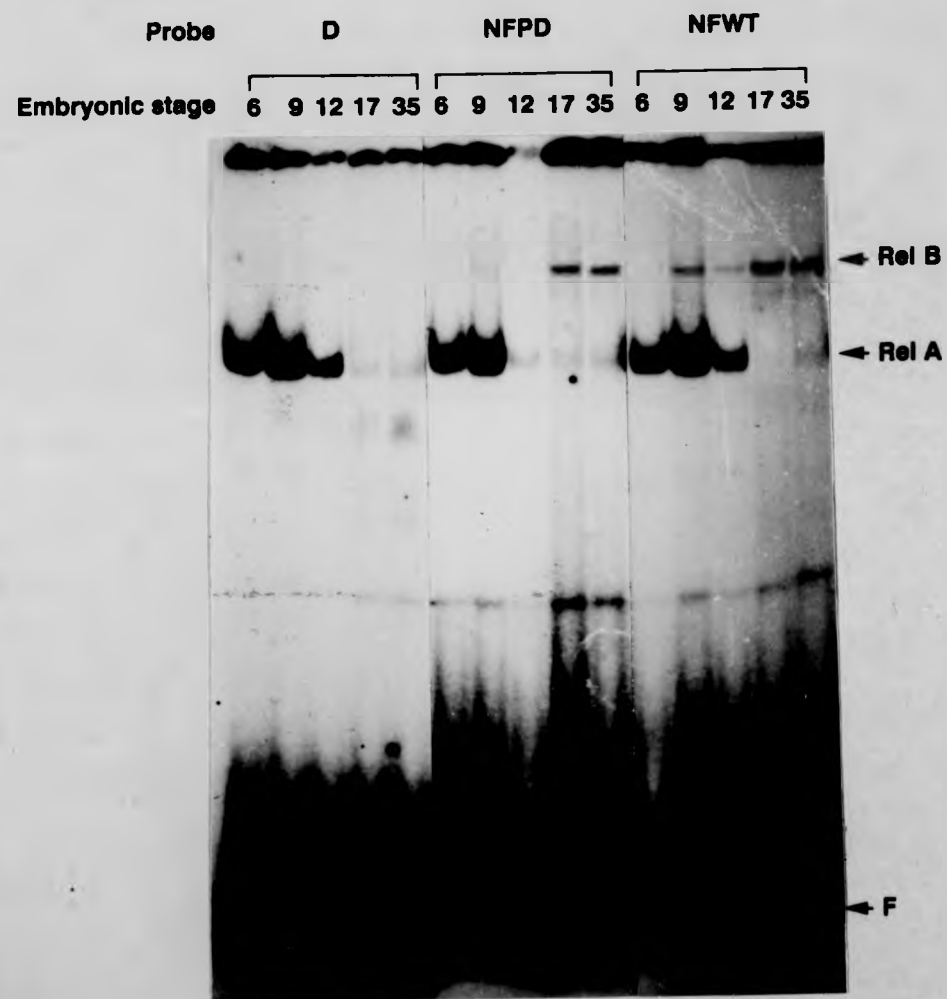
The binding activity of Rel A and Rel B was monitored, using band shift assays, through early X. laevis development by using extract from a range of embryonic stages (Stages; 6, 9, 12, 17, 26 and 35; figure 35). Rel A activity is detected at high levels in stage 6 embryos,

Figure 35.

Band shift assay showing temporal distribution of Rel A and Rel B binding activity in early X. laevis development.

Protein was extracted from X. laevis embryos at the following stages; stage 6 (early blastula), stage 9 (late blastula), stage 12 (gastrula), stage 17 (mid-neurula), stage 26 and stage 35. The three probes used all contained a consensus kB binding site (see figure G). The positions of Rel A, Rel B and free probe (F) are indicated. An unlabelled competitor binding site was not used in these assays.





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after which it peaks at stage 9 (late blastula) and then decreases to a moderate level at stage 12. After stage 12 (late gastrula), there is a significant decrease and only low levels of activity are detected later in embryogenesis (stages 17 and 35). In contrast, Rel B is first detected at stage 9, increases at neurula stage (stage 17) and this level is maintained in later (stage 35) embryos. From its temporal distribution, it is conceivable that Rel A may play a role in Xenopus, that is analogous to that of dorsal in Drosophila. Rel A and Rel B binding activities were shown in the last section to be detected in oocytes and therefore these proteins represent maternal genes, like dorsal. It seems that the binding activity of Rel B decreases between the oocyte stage and stage 6 before increasing again after MBT, which could indicate that this protein has two functions. The peak binding activity of Rel A precedes gastrulation, occurring between stage 6 and stage 9 (early blastula); dorsal protein is most abundant before cellular blastoderm stage. Both rel A and dorsal proteins persist at a lower level until after gastrulation. Rel A does not, however, bind more strongly to the dorsal binding site, when compared to wild-type and palindromic NFkB sites. Furthermore, if Rel A were like dorsal, one might predict that the temporal expression of Xenopus homologues of the dorsal target genes, twist and snail, xtwi and xsna respectively, to coincide with that of Rel A. Assuming that the expression of the protein reflects the

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level of transcripts, xsna and xtwi begin to increase at stage 10 (early gastrula; Sargent et al., 1989) and xtwi peaks at gastrula/early neurula (Hopwood et al., 1990). The increase in expression of these genes could therefore be in response to the activity of Rel A. Whether or not Rel A is involved in the activation of xtwi or xsna, the peak activity of Rel A occurs before gastrulation, so it is conceivable that it is involved in DV axis formation, even though it is not necessarily the Xenopus homologue of dorsal.

Rel B binding activity was detected in the nucleus of oocytes as well as the cytoplasm and therefore represents a maternal gene product like dorsal in Drosophila. The binding activity of Rel B does not correlate to the expression pattern of dorsal protein observed in Drosophila. However, the increase in binding activity does correspond to the time of transcription of known Xenopus homologues (xtwi and xsna) of dorsal target genes (twist and snail).

11.5 Spatial distribution of Rel A and Rel B in early X. laevis embryogenesis.

In order to determine whether Rel A and Rel B were located to a particular region of the embryo, band shift extract was prepared from dissections of embryos. Stage 9 embryos were dissected into animal, vegetal and equatorial regions; stage 10 embryos were dissected into dorsal,

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middle and ventral regions; stage 13 embryos were dissected into anterior, middle and posterior regions; stage 19 embryos were dissected into four approximately equal size sections, A,B,C and D where D was the most anterior. Equivalent amounts of protein from each dissection were used in these band shift assays (figure 36).

Rel B binding activity could not be detected using X. laevis dissected samples in band shift assays. It is possible that Rel B protein is fairly unstable and since there is a longer period of preparation of extract from dissected embryos, this protein may be degraded during preparation. Rather than degradation, this could be due to instability as a result of lower protein concentration in these extracts. The loss of Rel B activity was also observed in dissected oocytes. In contrast, Rel A binding activity was detected to some extent in all dissected samples. Rel A binding activity was detected to a similar extent for dorsal, middle and ventral regions of stage 10 (early gastrula) embryos. Dorsal protein is evenly distributed along the DV axis of Drosophila embryos, but is only located in the nucleus on the ventral side of the embryo. It has not been shown whether band shift assays using whole Drosophila cells would result in an overall ventral to dorsal gradient of binding activity, but comparisons have been made to the situation with NF-kB, where cactus is thought to play a role in preventing nuclear translocation similar to IκB in vertebrates, and

Figure 36.

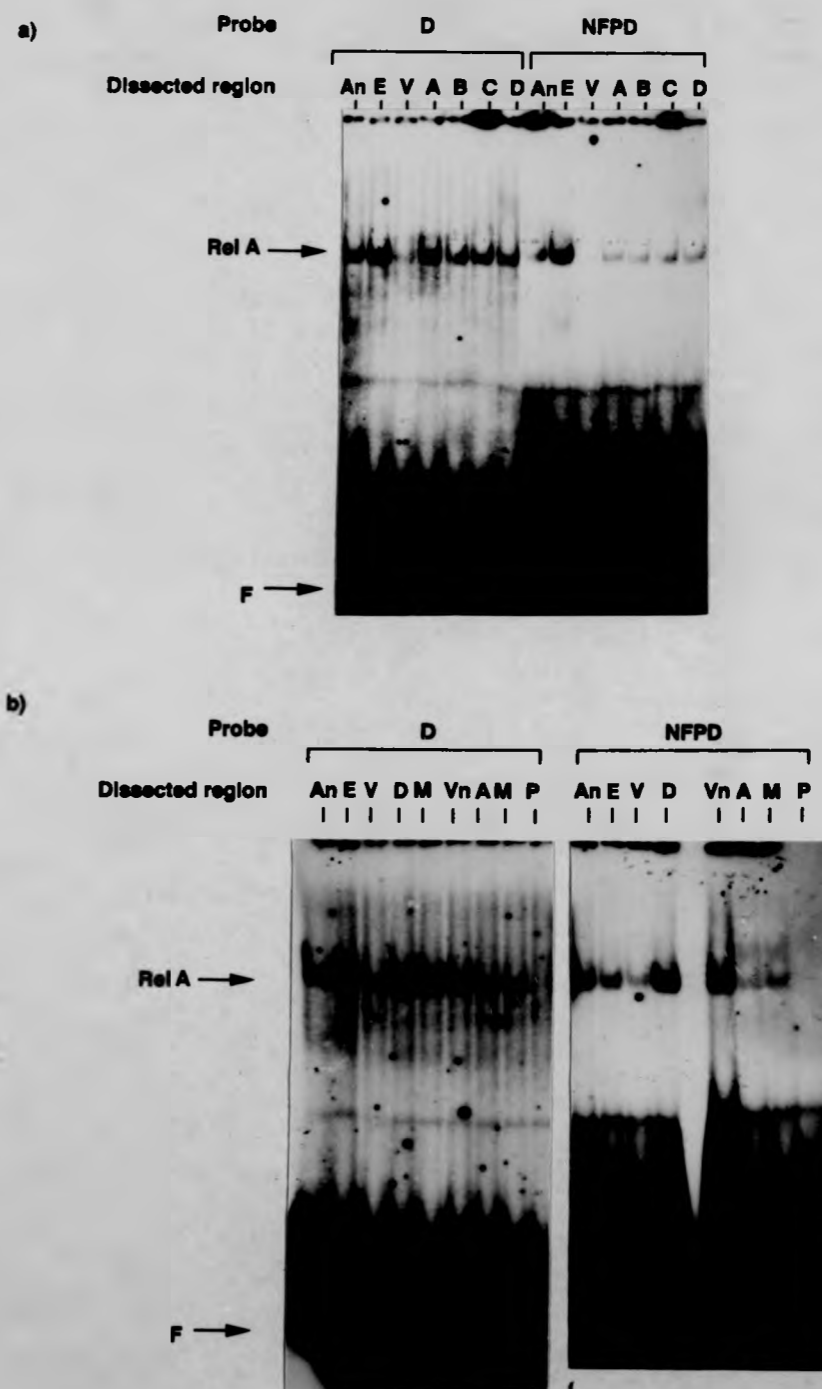
Band shift assays showing the spatial distribution of Rel A activity.

The two probes used both contained a consensus kB binding site (see figure 34a). The position of Rel A and free probe (F) are indicated. The amount of protein in each dissected sample was measured and the equivalent to one half on a whole embryo was loaded for each assay.

a) Protein extracted from the animal, equatorial and vegetal regions was from stage 9 (late blastula) embryos. Protein that was extracted from the A, B, C and D regions was from stage 19 embryos.

b) Protein extracted from the animal, equatorial and vegetal regions was from stage 9 (late blastula) embryos. Protein extracted from the dorsal, middle and ventral regions was from stage 10 (early gastrula) embryos. Protein extracted from the anterior, middle and posterior regions was from stage 13 (late gastrula) embryos.

An - Animal
E - Equatorial
V - Vegetal
D - Dorsal
M - Middle
Vn - Ventral
A - Anterior
M - Middle
P - Posterior



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also in preventing dorsal binding to DNA (see Introduction section 1.3.4). In this case a dorsal-ventral gradient of dorsal binding activity may be expected. It is not possible to say, therefore, whether or not the distribution of Rel A binding activity, detected in whole cell extract, is equivalent to the distribution of dorsal binding activity in the Drosophila embryo. Rel A present in extract from cytoplasm of dissected samples may be able to bind under these assay conditions. Consequently, an even binding distribution does not necessarily reflect an even nuclear-cytoplasmic distribution of Rel A. This equal distribution of Rel A protein was not apparent for the other sets of dissections. These differences were not due to the differing amounts of total protein present in each section as a result of the dissections since the protein concentration of extract prepared from each section was equalised in an assay.

Rel A binding activity was detected most strongly in the animal and equatorial regions and least strongly in the vegetal region of stage 9 embryos. Rel A binding activity was detected in the anterior and middle sections of stage 13 embryos, but only trace amounts of activity were detected in the posterior section. This uneven distribution of binding activity was lost at later stages. The level of binding activity of Rel A detected was the same for each part of stage 19 embryos, when dissected into four equal sections.

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Considering the even dorsal-ventral distribution and uneven anterior-posterior (and animal to equatorial) distribution of Rel A binding activity, a role may be played by Rel A in the formation of more anterior tissues. The temporal distribution of Rel A binding activity, which precedes gastrulation, may fit with the timing of the formation of the anterior-posterior axis, which occurs during gastrulation. A further indication that Rel A does not have a function similar to dorsal in Drosophila is that the distribution of transcripts of Xenopus xtwi and xsna, does not correspond to that of Rel A. Xsna is located predominantly in the vegetal half of embryos before MBT and at stage 12, its expression is increased fourfold in the DMZ, compared to the VMZ and it is also expressed later in the neural crest (Sargent and Bennett, 1990). Xtwi, first appearing in early gastrulae, is present only in the mesodermal cells, its distribution reflecting the subdivision of the mesoderm (it is expressed in all mesoderm except muscle, but is later also expressed in the neural crest ; Hopwood et al., 1989).

If Rel A does, indeed, have a role in AP axis formation, its distribution may be expected to reflect the distribution of a Xenopus homeobox gene that has been implicated in the formation of anterior and posterior tissues. Homeobox genes that have been implicated in the specification of anterior-posterior tissues include for example; Xhox3, Xlhbox6 and Xhox36 (Ruiz i Altaba and

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Melton, 1989c; Sharpe *et al.*, 1987; Condie and Harland, 1987; also see table 3). None of these genes have a distribution which matches the distribution of Rel A. UV-treated embryos (i.e. posteriorised embryos) were shown to have a fivefold increase in the level of expression of Xhox3 (Ruiz i Altaba and Melton, 1989b); perhaps anteriorised lithium-treated embryos would show a similar increase in the level of Rel A expression. The fact that the binding activity of Rel A was at a constant level for each of the four parts of dissected embryos at stage 19, but its distribution at stage 9 and 13 was uneven, could indicate dual roles for this protein in development.

Chapter 12.

The binding activity of x-rel protein.

12.1 X-rel encodes a binding activity apparently distinct from Rel A and Rel B.

To determine whether x-rel protein bound to kB-like sequences, band shift assays were initially carried out using full-length x-rel protein produced in E. coli. Full-length fusion protein was synthesised from pet3a526 and truncated fusion protein (which lacks half of the putative DNA binding domain) was synthesised from pet3b231, as described in Section 8.2, for use in band shift assays. All three probes referred to in figure 34a, were used in GEMSA, to try to determine whether x-rel protein was able to bind to the consensus DNA binding sequences. E. coli protein extract, containing large amounts, as determined by Coomassie staining of SDS gels, of full-length and deleted x-rel protein, detected a non-specific band for all three probes and only E. coli proteins that were specific (data not shown). Although x-rel fusion protein kB binding activity was not detected in bacterial extract, it does not necessarily follow that exogenous x-rel binding activity could not be detected in X. laevis oocyte/embryo extract, following injection of the message. The failure of x-rel protein to bind to kB sites in bacterial extract, given that the method used has been shown to work for another X. laevis transcription factor (see Smith and Old, 1991), could be due to it not being produced in a native form or

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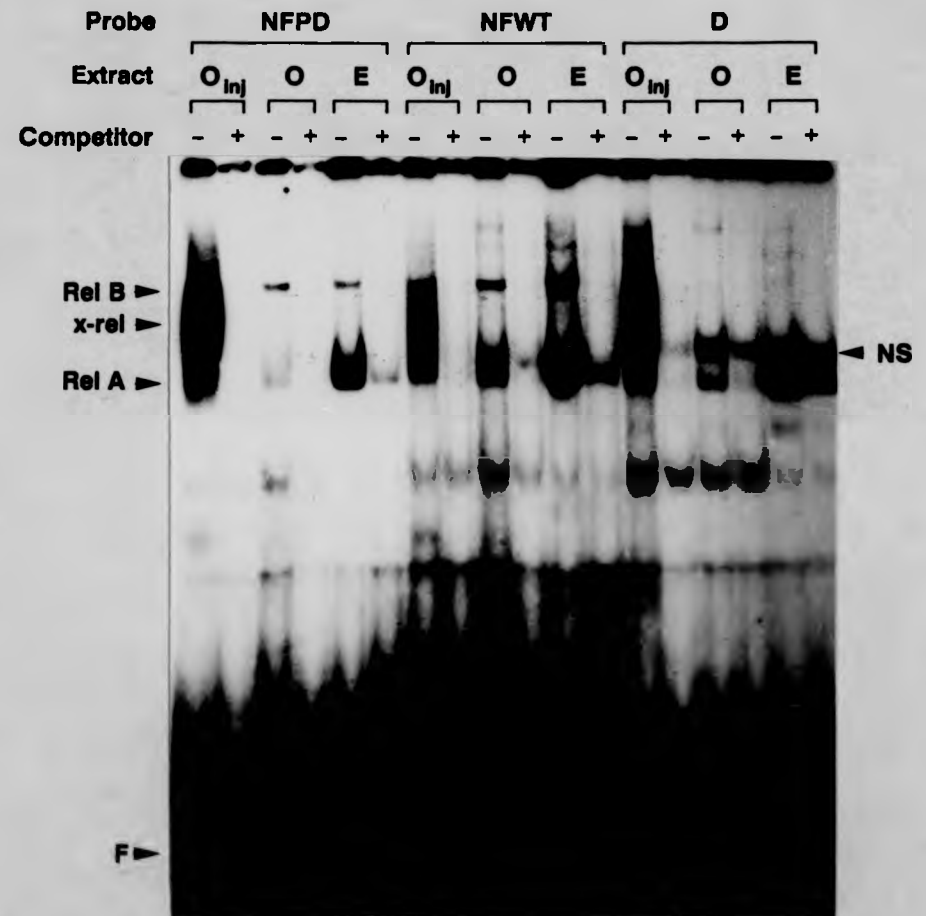
the requirement of other proteins to enable it to bind. It is also possible that it requires a secondary modification such as phosphorylation.

In order to establish whether endogenous x-rel binding activity could be detected in early embryogenesis, extract was prepared for band shift assays from X. laevis oocytes and embryos that had been injected with synthetic capped x-rel transcripts to give an idea of the mobility of the band expected. For this experiment, only RNA made from x-relSP64T was injected into oocytes, and subsequently band shift extract was prepared and its binding activity compared to that of uninjected oocytes and embryos. The resultant autoradiograph is shown in figure 37. A diffuse band, possibly representing several sub-bands, was detected from the injected oocyte extract. The mobility of this band could indicate that neither Rel A nor Rel B represent endogenous x-rel. However, either Rel-A or Rel B could represent a complex containing x-rel; this could be a heterodimer of endogenous x-rel with either a smaller (which would give rise to Rel A) or larger protein (which would give rise to Rel B). Mammalian p65, which is 77.5% (or 85.1% if allowing for conservative changes) similar over the N-terminal region (containing the putative DNA binding and dimerisation domains) to x-rel, does not occur naturally as a homodimer, and normally occurs as a heterodimer, p50-p65. However, overexpression of p65 can result in homodimers forming which can bind to some extent

Figure 37.

Band shift assay showing that x-rel encodes a binding activity that is possibly distinct from Rel A and Rel B.

Band shift extract was prepared from oocytes that had been injected with capped synthetic message produced from x-relSP64T (O_{inj}) and then incubated in Barth X for 24 hours. Protein was also extracted from non-injected oocytes (O) and stage 11 (mid gastrula) embryos (E). Three probes, each containing the kB enhancer consensus binding site (see figure 34), were used in this band shift assay. An excess of unlabelled probe was added to one of each pair of binding reactions, as competitor. The positions of x-rel protein, Rel A, Rel B and free protein are indicated.



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and this binding can just about be detected. Hence, if x-rel protein was like p65 in this respect, one would not expect to detect an endogenous x-rel band. Alternatively, the difference in size between exogenous x-rel and Rel A/Rel B could be the result of some form of processing or modification.

It is also possible that the diffuse nature of the band from the injected embryos could represent some activity of exogenous x-rel dimerising with other endogenous X. laevis rel-related proteins, perhaps in a situation analogous to p50-p65. p50 dimers can bind to DNA, but they do not occur commonly; most p50 is present in the heterodimer, p50-p65.

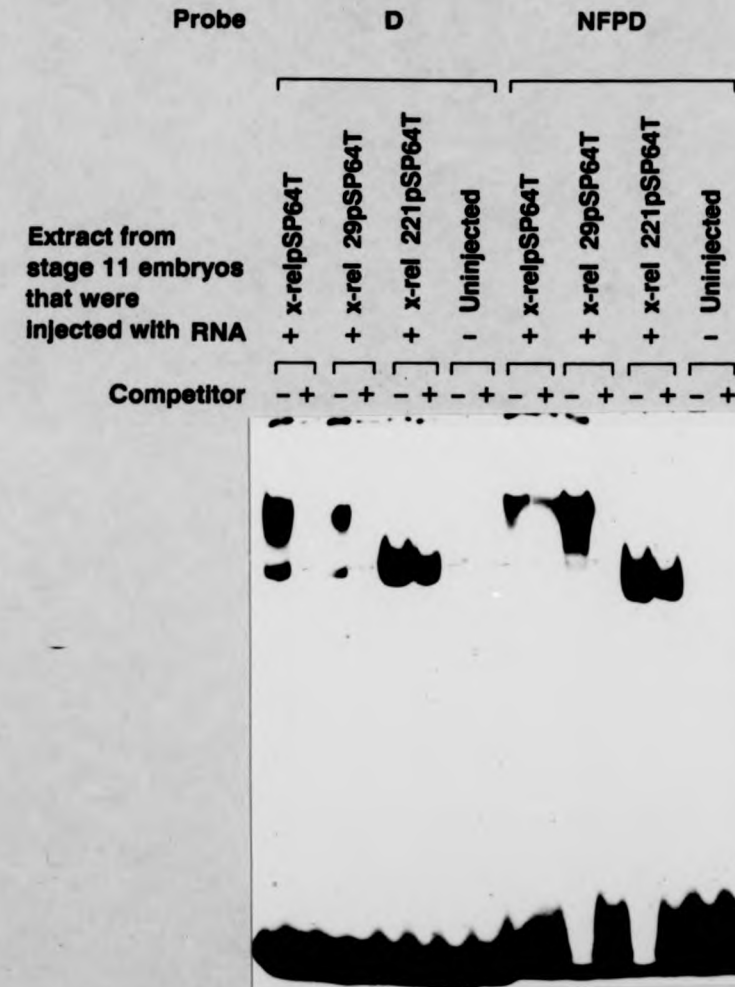
Assuming that the distribution of x-rel protein reflects that of its message, it does not correspond to the distribution of the Rel B binding activity. X-rel message peaks at stage 9 and then falls until only low levels are present after stage 12; Rel B binding activity is first detected at stage 9 and increases at stage 17. The distribution of x-rel message is fairly similar to the distribution of the Rel A binding activity. In both cases, they are present at stage 6, their peaks occur at stage 9 and they decrease to quite a low level after stage 12, but expression is maintained in later stages. This is consistent with the idea that x-rel may be a component of Rel A (that is, in a heterodimer with a lower molecular weight protein) and this possibility cannot be excluded.

Rel A appears to have equal affinity for all three

Figure 38.

X-rel, x-relΔ29 and x-relΔ221 exogenous protein all bind to kB DNA binding sites.

Band shift extract was prepared from stage 11 embryos that had been injected with capped synthetic message produced from x-relSP64T, x-relΔ29pSP64T and x-relΔ221pSP64T. Protein was also extracted from stage 11 non-injected embryos. Two probes, D and NFPD, each containing the kB enhancer consensus binding site (see figure 34), were used in this band shift assay. An excess of unlabelled probe was added to one of each pair of binding reactions, as competitor.



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probes, whereas Rel B apparently prefers a more NF- κ B-like than dorsal-like binding site. p50-p65 heterodimers prefer to the wild-type binding site (NFWT), but (p50)₂ and (p65)₂ homodimers prefer the palindromic site (NFPD; see Introduction Section 1.3.3). The situation with x-rel may be analogous.

In a second experiment protein extract was made from stage 11 (early gastrula) embryos that had been injected with x-rel, x-relΔ29 and x-relΔ221 pSP64T transcripts, for use in GEMSA (figure 38). Each of the respective x-rel proteins contained in these extracts was shown to bind to the κ B consensus sequence contained in the D and NFPD probes. The fact that the x-relΔ221 protein, which contains all of the putative DNA binding domain but lacks the putative activation domain, can bind to the κ B sites and so may encode a dominant negative mutant, may be particularly important in explaining the different phenotype produced by injection of this message (described in Chapter 13) compared to the wild-type message.

One way of establishing whether an exogenous protein corresponds to another protein, identified in GEMSA, is to use antiserum raised against the exogenous protein. If the two proteins are related, the band representing the binding activity is abolished and/or shifted higher up the gel as a result of binding to the antibody, known as a 'supershift'. The antiserum raised against pet3b231 was previously shown (see Section 10.2) to have activity to x-rel proteins by

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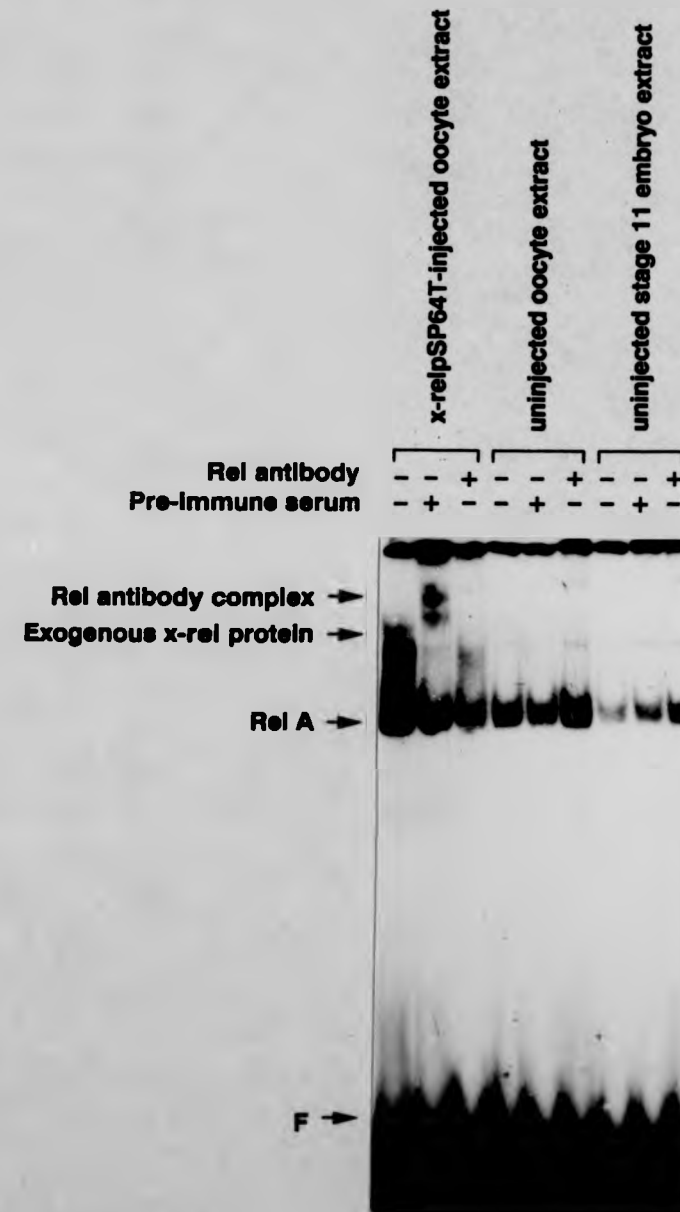
staining sections of oocytes, that had been injected with x-rel message, with the antibody and then counter staining with a fluorescently labelled anti-immunoglobulin. Band shift assays were carried out where the antiserum raised against pet3b231 fusion protein was added to binding reactions containing extract prepared from injected and uninjected oocytes or embryos. The results are shown in figure 39. Although the antiserum has abolished the binding activity of the exogenous x-rel protein translated from exogenous mRNA, no supershift of an antibody-x-rel protein complex was observed. Furthermore, binding activity of x-rel exogenous protein was abolished by the addition of pre-immune serum (and extra bands of lower mobility observed). Neither the antibody nor the pre-immune serum affected the binding activity of Rel A or Rel B. Although it would be dangerous to draw conclusions from this experiment, which clearly did not work in the manner anticipated (i.e. antiserum, and not pre-immune serum abolishing x-rel binding) it could be argued that since x-rel binding was abolished and Rel A and Rel B were not, that x-rel is unlikely to be a component of Rel A or Rel B.

The antibody may not react with endogenous or exogenous x-rel protein due to the method of preparing the fusion protein it was raised against. pet3b231 was purified from an SDS gel (see Section 9.2) and it is possible that the antibody raised from it only reacts with SDS-protein complexes. The sections of injected oocytes that were

Figure 39.

Band shift assays showing that x-rel may not correspond to Rel A or Rel B as determined using antiserum raised against part of x-rel.

Band shift extract was prepared from oocytes that had been injected with capped synthetic message produced from x-relSP64T (O_{inj}) and then incubated in Barth X for 24 hours. Protein was also extracted from non-injected oocytes (O) and uninjected stage 11 embryos (E). Assays in lanes 2, 5, 8, 11, 14 and 17 contain anti x-rel protein (raised against the pET-3b231 fusion protein). Assays in lanes 3, 6, 9, 12, 15 and 18 contain anti x-rel pre-immune serum. Lane 19 contains no protein extract to show that the sera does not bind non-specifically. Two probes, both containing the kB enhancer consensus binding site (see figure 34), were used in this band shift assay. The positions of x-rel, Rel A, Rel B and free probe (F) are indicated.



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stained with antibody and produced a positive reaction, were treated with SDS.

In view of the above findings it is, in my opinion, unlikely that either Rel A or Rel B represent endogenous x-rel. There are several reasons why endogenous x-rel protein may not have been detected. It is unlikely that x-rel protein is not expressed in the stages of embryos that were analysed; although it is possible that some translational control is occurring, the temporal pattern of RNA expression would usually roughly indicate the temporal pattern of protein expression. However, although band shift assays are a sensitive method of detecting binding proteins, it is possible that the amount of endogenous x-rel protein is below the minimum amount that can be detected by this method. X-rel mRNA is very difficult to detect, and is certainly orders of magnitude less abundant than the injected, exogenous message.

The failure to detect endogenous x-rel protein by its binding activity may have been the result of anchoring of the endogenous protein by a cytoplasmic inhibitor protein, in a situation analogous to p50-p65-IkB (see Introduction Section 1.3.4). Binding activity would therefore only be detected when large amounts of x-rel protein were synthesised by injecting synthetic x-rel message and the binding sites of the inhibitor protein were saturated. It has been shown that treatment of the cytosolic fraction from unstimulated cells of a pre-B cell line, with

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dissociating agents can activate the DNA binding activity of NF- κ B-like proteins (Baeuerle and Baltimore, 1988). The addition of dissociating agents, such as deoxycholate (DOC), disrupts the association of the p50-p65 dimer with the inhibitor protein I κ B, allowing binding to be detected in cytosolic band shift extracts (see Introduction section 1.3.4). I κ B binds to the p65 protein, thereby retaining it in the cytoplasm; when this association is disrupted, p65 is free to be translocated to the nucleus and bind, as a heterodimer (p50-p65), to DNA. To try to detect endogenous x-rel protein, band shift assays were therefore carried out using embryo extract in the presence of 0.6% DOC, 1.2% NP40 (conditions described in Baeuerle and Baltimore, 1988), which dissociates the NF- κ B-I κ B complex, allowing the dissociated proteins to bind to DNA. This method of dissociating proteins was not successful in detecting endogenous x-rel protein (data not shown).

Alternatively, all of the endogenous x-rel may have been detected as a component of Rel A or Rel B binding activity. As argued earlier, the possibility exists that x-rel could be a component of Rel A or Rel B (more likely Rel A on the basis of their temporal distribution). To determine whether this is the case, further antibodies could be raised against x-rel fusion proteins for use in GEMSA. A C-terminal antibody would be most informative, since this is likely to be x-rel-specific and not cross react with other members of the rel family. More information on the

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spatial distribution of x-rel message so that this could be compared to the spatial distribution of Rel A would be of use. Other forms of band shift assays, involving proteolytic clipping (PCBSA), could be used to compare the pattern of bands between Rel A and x-rel and therefore give an indication as to whether x-rel is a component of Rel A. Finally, x-rel and Rel A could be cross-linked to internally labelled band-shift probe in the binding reaction, isolated from a band-shift gel, and the now labelled proteins resolved by SDS-PAGE. If Rel A contained x-rel, a band of the same mobility would be observed (this method has been described for other rel family proteins, see Ballard *et al.*, 1990)

12.2 Dimerisation of wild-type and mutant x-rel proteins.

The DNA binding domain identified in rel family members has also been shown to be a dimerisation domain for some members. GEMSA studies using kB-binding proteins, in particular, indicate that these proteins usually bind as homo- or heterodimers. NFkB itself is composed of a heterodimer of two rel-like proteins, p50 and p65. It is conceivable that x-rel homodimerises or heterodimerises with another X. laevis rel-related protein and that this association is essential for its natural function (see Introduction 1.3.3).

In order to establish whether x-rel could homodimerise, the ability of full-length x-rel protein to dimerise with

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truncated x-rel proteins was investigated. Band shift assays were carried out where extracts, made from oocytes that had been injected with synthetic transcripts made from x-relpSP64T and x-relΔ221pSP64T, were mixed in vitro. The resultant autoradiograph is shown in figure 40a. When the afore mentioned extracts were mixed in vitro, no bands of intermediate mobility, compared to non-mixed extracts, were detected.

Data from GEMSA studies on NF-κB, indicated that little binding activity for p50-p65 was detected if p50 and p65 were renatured separately and then mixed, but combined renaturation resulted in an increase of p50-p65 binding activity (Urban et al., 1991). This may be because the formation of p50-p65 heterodimers cannot occur if homodimers already exist. Band shift assays were therefore carried out using extract made from oocytes that had been co-injected with synthetic message made from x-relpSP64T and x-relΔ221pSP64T. This may allow oligomerisation to occur as proteins are co-synthesised. The result is shown in figure 40b. A band of intermediate mobility was detected from the extract made from oocytes that had been co-injected with the wild-type and truncated x-rel transcripts.

This indicates that two molecules of x-rel protein must bind to the band-shift probe, probably as a result of dimerisation (by analogy to other rel family members). The observation that mixing of extract from x-relpSP64T

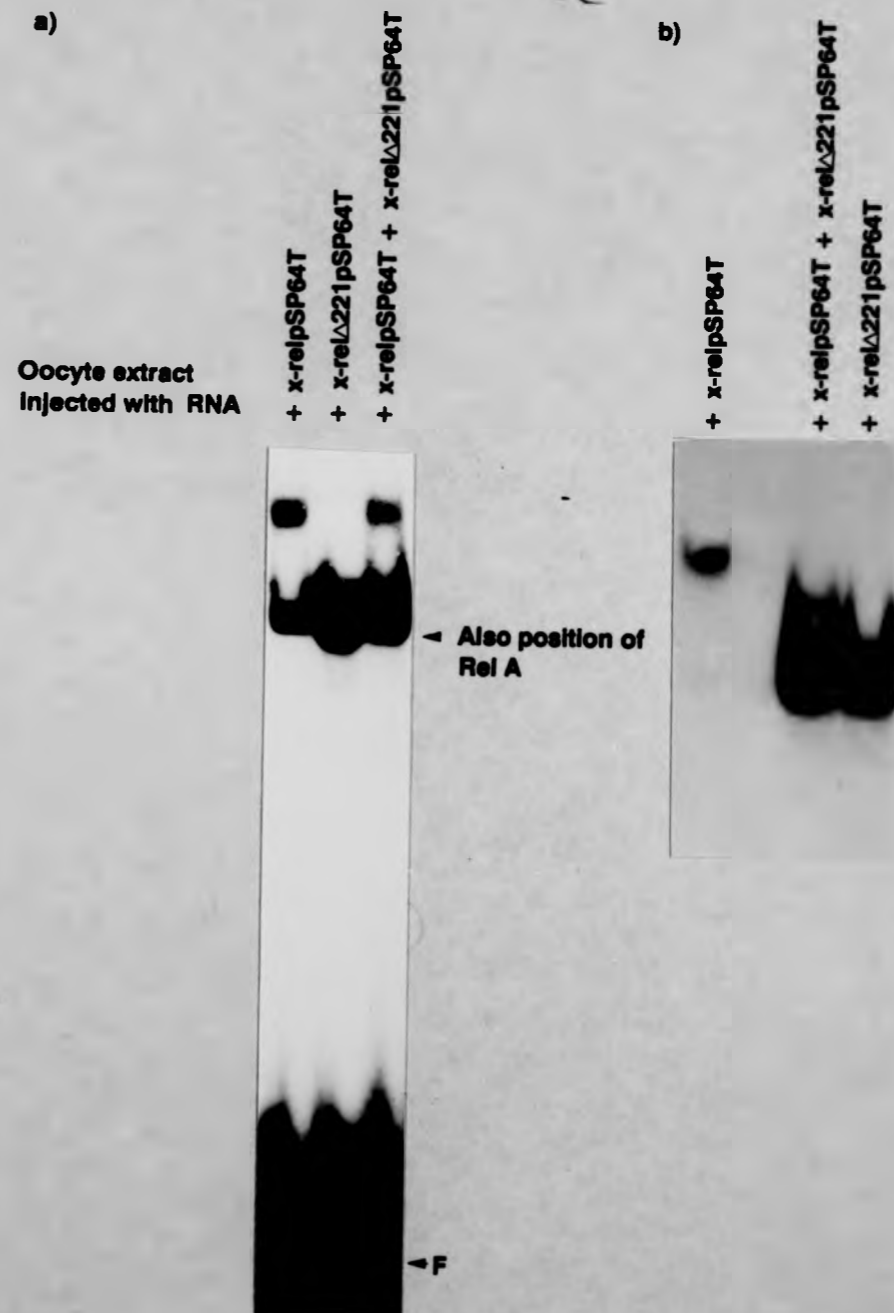
Figure 40.

Band shift assay showing dimerisation of x-rel protein with x-rel Δ 221 protein.

Capped synthetic RNA was made from x-relpSP64T and x-rel Δ 221pSP64T, using SP6 RNA polymerase. These transcripts were injected into oocytes, which were then incubated in Barth X for 24 hours and protein extracted from them. Only the D probe was used in these assays.

a) Band shift assays using protein extract prepared from oocytes, which had been injected with the transcripts indicated, and then mixed in vitro.

b) Band shift assays using protein extract prepared from oocytes, which had been co-injected with the transcripts indicated. The dimerising proteins are indicated (D).



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message-injected and x-rel $\Delta 221$ pSP64T message-injected oocytes did not result in an intermediate band, indicates that it is, in fact, dimerisation which is taking place. These observations suggest that x-rel and x-rel $\Delta 221$ homodimers form in injected oocytes, and do not dissociate to an appreciable extent during the GEMSA binding reaction, so that heterodimers can form. However, when co-injected x-rel and x-rel $\Delta 221$ heterodimers form. This is analogous to the situation in NF-kB, where co-renaturation of components is required for dimerisation. Heterodimerisation, as well as competition for binding sites, is a mechanism by which x-rel $\Delta 221$ may act as a dominant negative mutant (see Chapter 13). Endogenous x-rel protein, is therefore, likely to occur as a dimer, although not necessarily a heterodimer.

The fact that an intermediate band is seen on co-expression of x-relpSP64T and x-rel $\Delta 29$ pSP64T messages is of some importance to the argument that x-rel may be a component of Rel A. If this were the case, it would be anticipated that (eventhough x-rel $\Delta 221$ has the same mobility as Rel A) an endogenous x-rel/exogenous x-rel $\Delta 221$ intermediate band, and a band corresponding to x-rel $\Delta 221$ heterodimerising with the other, hypothetical, component of Rel A (smaller than Rel A and x-rel $\Delta 221$ bands) would be observed. These bands are, in fact, never observed (see figures 38 and 40). This strongly suggests that x-rel is not a component of Rel A. However, co-translation of

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exogenous x-rel messages and endogenous Rel A, may be required for mixed dimers to form (see above). If Rel A is synthesised early in oogenesis and after synthesis of exogenous x-rel protein in injected embryos (strong expression of Rel A begins at late blastula), co-translation of Rel A components and exogenous x-rel protein may not occur. Clearly, the experiments suggested earlier (see Section 12.1) are required to establish whether or not x-rel is a component of Rel A.

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Chapter 13.

Phenotype observed as a result of injecting synthetic x-rel message into embryos.

13.1 Introduction.

In Xenopus, there is difficulty in ascertaining the function of any cloned gene which may have a role in early development mainly because of the difficulty in producing transgenic animals. Transgenic animals have been produced from Xenopus (Etkin and Pearman, 1987), but they are mosaic in the number of copies of exogenous sequence that are integrated and the tissues in which expression is found. Furthermore, the long reproductive cycle of Xenopus is a disadvantage in analysing gene function by breeding transgenic animals. In contrast, transgenic mice can be produced relatively easily and gene disruption is possible by gene targeting in totipotent embryo-derived stem (ES) cells, which can lead to the generation of mice with a desired genotype (reviewed by Capecchi, 1989; Westphal and Gruss, 1989). On the basis of homologies with other proteins in other organisms, such as Drosophila, related genes have been identified in the mouse. Pax-1, for example, was isolated as a potential developmental control gene, on the basis of its homology to the Drosophila paired-box which was originally identified in a subset of segmentation genes known to play critical roles in

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Drosophila development (Akam et al., 1987). Murine pax-1 has been strongly correlated with a mouse mutation identified at the undulated (un) locus (chromosome 2; Balling et al., 1988). Homozygous mice show vertebral abnormalities over the entire length of the vertebral column, a consequence of perturbation in the position of the boundary between anterior and posterior sclerotome halves in the mid-gestation embryo. Transgenic mice that have mutations in Pax-1 gene structure or expression in three independent un alleles (Balling et al., 1988; Kessel and Gruss, 1990), display vertebral abnormalities. The restriction of Pax-1 expression to posterior sclerotome halves during normal vertebral development (Deutsch et al., 1989) suggests a causal role for Pax-1 in the generation of the un phenotype and perhaps a directive role in the sclerotome determination.

In addition to the impracticability of producing transgenics, the relatively long life cycle of Xenopus, makes it impractical to rear large numbers of individuals for the many generations required for other methods of genetic analysis. In Drosophila, it is possible to screen for all genes that can mutate to produce a given phenotype, on the basis of viability, aspects of morphology or by using antibodies. Having identified these mutations, genetic complementation tests can be used to estimate the number of different genes involved in the process and phenotypes of individuals carrying mutations in more than

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one of the genes can be studied to infer hierarchies of gene function. An example of this approach is the identification of the genes involved in setting up dorsoventral polarity in the early Drosophila embryo (see Introduction Section 1.2.2). Using the information obtained from the phenotype, the gene of interest is then cloned. Cloning may either be carried out by chromosome walking in the region of the genome containing the desired gene, after classical genetic mapping has allowed determination of the position of the gene relative to known genes (Bender et al., 1983) or by chromosome dissection, where the desired gene is physically dissected from the polytene chromosomes of the salivary glands and used as a source of DNA for constructing a library in a bacteriophage lambda vector. Alternatively, transposon tagging may be used to biochemically tag a gene to aid in its cloning (Bingham et al., 1981). P transposable elements allow tagging that is under genetic control and can be manipulated experimentally.

Xenopus, despite not being amenable to the genetic techniques described above for mice and Drosophila, does have the advantage that the embryo is large, the fate map is known and it is relatively easy to micro-inject genes or their products into predetermined places early in development. One method of inactivating the function of a gene is at the RNA level, where antisense RNA is microinjected into embryos at the 2-cell stage. However, it

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is difficult to generate maternal mutations of developmentally important genes, since at least some signalling molecules, for instance, are derived from maternal transcripts and many of them are likely to have a function later in development. Furthermore, although success has been achieved with this method, for example the inhibition of translation of injected globin can be inhibited by injected antisense globin in oocytes (Melton, 1985), it has been shown that injected antisense RNA does not form stable hybrids in vivo with its complementary mRNA. This was thought to be due to a RNA duplex unwinding activity, which exists in eggs and early embryos (Rebagliati and Melton, 1985; Harland and Weintraub, 1985), but may be due to base modification (Bass and Weintraub, 1988; Wagner et al., 1989). Antisense oligonucleotides may provide a solution to this problem. Although unmodified oligonucleotides are very sensitive to cellular DNases, the recent use of phosphorothioate oligonucleotides (PTO's) has overcome this susceptibility. Furthermore, PTO's display similar hybridisation kinetics to unmodified oligonucleotides, are able to form a RNase H suited substrate and are taken up directly from the medium by cells (and can therefore be used directly in tissue culture; eg. Cazenave et al., 1989). A recent example of the use of PTO's was shown in mouse organ cultures; PTO inhibition of nerve growth factor receptor (NGFR) expression was shown to inhibit kidney morphogenesis,

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indicating that NGFR is involved in the differentiation of kidney tubules, in addition to its role in the development of the nervous system (Sariola et al., 1991). Injection of PTO's may prove useful in studying genes involved in Xenopus embryogenesis. However, PTO's will not be useful in studying genes which have a maternal protein store.

A knock-out experiment may also be achieved by injecting an antibody raised against the wild-type protein. An example of where this approach has been used is the injection of anti-long XlHbox1 protein, which resulted in specific malformations of the anterior spinal cord (Oliver et al., 1988).

As an alternative to producing a transgenic animal, gene products have been overexpressed in Xenopus, in their normal or ectopic positions, to try to ascertain their function. A recent example of this was shown by the overexpression of Xhox3 in the anterior region, where it is normally expressed at its lowest level, producing a series of axial defects that led to a failure in forming head structures (Ruiz i Altaba and Melton, 1989c). There was also a concomitant increase in the level of posterior markers, suggesting a role for Xhox3, in accordance with its natural posterior location, in the specification of posterior structures.

However, knock-out experiments may have advantages over overexpression; whereas overexpression as a result of injecting synthetic messages will generally be precocious,

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knock-out experiments will by their nature only work at the correct time. In addition, such knock-out experiments are more specific; homeoboxes have common DNA binding sites, and so a phenotype may represent disruption of another related protein or proteins. Furthermore, overexpression in the wrong cell type will not necessarily give the same result as endogenous expression in the correct cell type.

A knock-out experiment may also be achieved by inhibition of the wild-type gene by overproducing an inhibitory variant of the same product. A particular functional region of a protein, such as the DNA binding site or substrate binding site, could be mutated independently. Since many transcription factors bind as dimers (Mitchell and Tijan, 1988), and enzyme receptors interact via ligand induced oligomerisation (Schlessinger, 1988), a mutant derivative, which can interact with wild-type protein, but lacking in other functions, can be inhibitory by causing the formation of non-functional multimers. For example, a naturally occurring form of the FGF receptor, which lacks a cytoplasmic tyrosine kinase domain, but retains an intact extracellular domain and transmembrane domain, has been shown to inhibit FGF signalling in the oocyte (Amaya et al., 1991).

The analysis of the level of protein synthesis by injected x-rel messages has already been discussed in Sections 7.3 and 7.4. The natural pattern of expression of x-rel message (peaking during early gastrulation) dictated

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that protein from injected x-rel message should continue to be present throughout gastrulation in order to investigate the role of x-rel in development. This was achieved by the use of the pSP64T vector, which produces transcripts which are stable in Xenopus embryos. Per embryo, the level of x-rel expression from injected message obviously represented massive overexpression, although since endogenous x-rel expression may be limited to a subset of cells, it is more difficult to compare levels of expression in cells expressing endogenous and exogenous message. Overexpression may, therefore, be more a matter of the variability of the spreading of exogenous message to different regions of embryos overriding the variation in the quantities of proteins made with different mRNA doses.

Complications resulting from the overexpression of injected mRNA include its limited diffusion from the site of injection, compared to the rate at which it is compartmentalised by cell division. Generally, RNA is most concentrated in a broad region around the site of injection (Colman and Drummond, 1986). However, the diffusion rate should be sufficiently rapid and extensive to ensure that most of the daughters of the injected blastomere receive the injected RNA and this injected RNA is inherited by a wide enough range of cells to cover most body parts. However, one cannot eliminate the possibility that variations in a phenotype may result from a variable uneven distribution. Variations may also arise as a direct result

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of the injection process, whereby a small amount of cytoplasm leaks from the site of injection and forms a cytoplasmic ball containing some of the injected RNA.

In the next section of this Chapter, the phenotype data described is based on external appearance. However, histological sections of apparently normal embryos injected with a particular RNA sample show that they may also have internal defects. Consequently the % of embryos that are recorded as developing abnormally, is at the lower limit.

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13.2 Injection of x-rel, x-rel Δ 29 and x-rel Δ 221 messages produced from a pSP64T vector into X. laevis embryos and observations from phenotype.

13.2.1 Outline of injection experiments and characteristics of messages injected.

Message was synthesised using the SP6 promoter from three constructs; x-relpSP64T, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T, the cloning of which is described in Section 7.2. X-rel Δ 29pSP64T differs in its coding region from full-length x-rel by a deletion of 29 amino acids at the C-terminal end. This clone was made since it may result in altered nuclear localisation, since this occurred as a result of loss of residues from the C-terminus in certain other rel family members which are translocated to the nucleus after a similar deletion (eg. chicken c-rel and turkey v-rel; see Introduction section 1.3.4). A different phenotype resulting from the injection of these two clones might, therefore, have been expected since x-rel Δ 29 message may get translocated to the nucleus more quickly than x-rel in embryos. However, there is currently no evidence that proteins of the p65 NF-kB class have a cytoplasmic anchoring domain. The results showing translocation into oocytes and gastrula nuclei tend to argue against this (see Section 7.3 and 10.3), at least as an important regulatory point in early development. However, it could be argued that this hypothetical control

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mechanism is saturated.

X-rel Δ 221pSP64T produces a protein lacking all of the putative activation domain (which is 8.6% proline-rich and 14.9% serine-rich), a region of 221 amino acids. The abnormalities in the phenotype resulting from injection of this message is predicted to appear opposite to the messages described above. This is because x-rel Δ 221 may act as a dominant negative mutant, perhaps by dimerising to itself or to endogenous x-rel and forming non-active heterodimers that are capable of binding to DNA, but incapable of activating transcription from the target genes of endogenous x-rel homodimers and heterodimers. If endogenous x-rel does bind as a heterodimer, x-rel Δ 221pSP64T may titrate out wild-type endogenous x-rel protein, thereby preventing association with its related partner. Inhibition of transcription factors that belong to the rel/NF-kB family by a transdominant negative mutant of p50 has been shown (Logeat *et al.*, 1991). This mutant, Δ SP, lacks the DNA binding domain, but retains the dimerisation domain and is capable of forming homo- or hetero- dimers. Δ SP protein abolishes *in vitro* the DNA binding of p50, c-rel and v-rel wild-type proteins. Furthermore, it functions, *in vivo*, as a trans-acting dominant negative regulator; the transcriptional inducibility of the HIV LTR (which contains two potential NF-kB sites) by PMA is inhibited when it is co-transfected into CD4⁺ T cells (Logeat *et al.*, 1991).

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Δ Fos B is an example of a naturally occurring dominant negative mutation (Nakabeppu and Nathans, 1991). Δ Fos B retains the DNA binding and dimerisation domains of Fos B. In transfection assays, Δ FosB is able to inhibit the activation of transcription by Fos-Jun oligomers from an AP-1 site and the repression of the c-fos promoter by Fos B.

Loss of function of endogenous x-rel may thus be the outcome of x-rel Δ 221pSP64T injections and this would be reflected in the most serious, and perhaps the only, disruption of the embryo occurring in regions where x-rel is normally active. An alternative, although less likely, consequence of overexpression of x-rel Δ 221pSP64T is that it could titrate out an I κ B-like protein and activate a p50-like protein. In this case, wild-type and deleted proteins would generate the same defects. Clearly, much of this speculation would be resolved by investigating the effect of these transcripts on κ B-containing genes. This work is in progress.

In interpreting our data, we postulate that overexpression of x-relpSP64T and x-rel Δ 29pSP64T messages would be expected to produce a gain in function phenotype and most seriously disrupt the region of the embryo where x-rel is not normally active.

Controls used in the injection experiments described in following sections were usually uncapped message synthesised at the same time, from the same sample of DNA

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and using the same source of reagents. In this way, any aberrations of development occurring as a result of introduced message cannot be attributed to the quality or toxicity of the RNA, if injection of the uncapped RNA results in normal development. Uncapped RNA is the obvious control since it has all the properties of the capped RNA except that CAP analogue has not been added and so one would predict that it would not be as efficiently translated, compared to capped message, and it would also have lower stability. It was shown in Section 7.4 that translation could not be detected as a result of injection of uncapped x-rel message into embryos. The data from these experiments should therefore provide some indication of the function of x-rel in early Xenopus development. In some experiments we used 'anti-sense' transcripts, in which the globin 3' and 5' strands are from the sense strand, but the x-rel coding region is anti-sense. In addition, to the extent that x-relSP64T and x-relΔ221pSP64T messages generate different phenotypes, they control each other.

13.2.2 Overall observations of phenotype observed after injection of x-relpSP64T, x-rel Δ29pSP64T and x-rel Δ221pSP64T messages.

Capped and uncapped synthetic transcripts were produced from the x-rel constructs; x-relpSP64T, x-relΔ29pSP64T and x-relΔ221pSP64T and 20nl of a range of concentrations of the respective RNA was injected, by bipolar injections,

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into each blastomere of embryos at the 2-cell stage. The results of these injections are summarised in table 6. This data is based on pooled experiments, which obscures the cleanness of the effect to some extent and obscures the dose-dependent effect. Different preparations of synthetic message produced a different degree of potency. These differences could have been due to the inefficiency of capping. With this proviso, two independent clones for wild-type x-rel message were used which produced exactly the same phenotype over the same range of concentrations, suggesting that the differences in the preparation of RNA result from different doses rather than the message causing a different effect (eg. through mutations introduced when making the constructs).

The main conclusions from these experiments is that there is a direct correlation between concentration of message injected and the % of embryos that develop normally. Injection of 500ug/ml capped x-relSP64T message results in 100% abnormal embryos, whereas 52% are normal after injection of 50ug/ml capped x-relSP64T message. Although only 66% of embryos injected with 500ug/ml uncapped x-relSP64T message develop normally, a further 19% appear to have 'spina bifida', which is not related to the presence of the particular message, but is a common effect observed after injection of any synthetic RNA or DNA. 'Spina bifida' embryos, in our jargon, represent the failure of the blastopore to close and the posterior axis

Table 6.

Summary of scored embryos, showing stage-related abnormalities, after injection with x-relpSP64T, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T messages at the 2-cell stage.

Message from the pSP64T constructs was injected at the concentration indicated. Numbers recorded are based on pooled experiments.

RNA sample	No. of expts.	No. injected	Cleaving normally		Blocked at stage 10-10.5		Normal development		Later abnormal development		Spina bifida	
			No.	%	No.	%	No.	%	No.	%	No.	%
500ug/ml x-relpSP64T	2	49	46	94	45	98	0	0	1	2	0	0
250ug/ml x-relpSP64T	3	68	63	93	36	57	2	3	25	40	0	0
100ug/ml x-relpSP64T	5	113	100	88	39	39	2	2	59	59	0	0
50ug/ml x-relpSP64T	3	70	63	90	1	2	29	46	33	52	0	0
500ug/ml uncapped x-relpSP64T	5	98	92	94	0	0	65	71	7	8	20	22
100ug/ml uncapped x-relpSP64T	2	44	34	80	0	0	32	94	0	0	2	6
500ug/ml antiense x-relpSP64T	2	41	39	95	0	0	33	85	1	3	5	13
500ug/ml x-rel 29pSP64T	4	90	72	80	15	21	4	6	53	74	0	0
250ug/ml x-rel 29pSP64T	3	63	51	81	12	24	5	10	32	63	2	4
100ug/ml x-rel 29pSP64T	2	49	42	86	0	0	13	31	29	69	0	0
50ug/ml x-rel 29pSP64T	1	25	16	72	4	22	9	50	5	28	0	0
500ug/ml uncapped x-rel 221pSP64T	4	88	73	83	1	1	58	79	2	3	12	16
500ug/ml x-rel 221pSP64T	2	41	34	83	0	0	0	0	25	74	9	26
250ug/ml x-rel 221pSP64T	3	59	54	92	0	0	2	4	48	89	4	7
100ug/ml x-rel 221pSP64T	2	40	36	90	0	0	17	47	19	53	0	0
50ug/ml x-rel 221pSP64T	2	39	36	92	0	0	21	58	9	25	6	17
25ug/ml x-rel 221pSP64T	1	20	17	85	0	0	12	71	5	29	0	0
500ug/ml uncapped x-rel 221pSP64T	2	40	38	95	0	0	36	95	2	5	0	0

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develops in twin fashion on each side of the open blastopore; heads are usually normal, but tails may be absent or very stunted. 'Spina bifida' embryos are, therefore, most safely regarded as normal for the purposes of these experiments.

As shown in tables 6, 7 and 8, there is a large variation in abnormality within the same batch of embryos that have been injected with a particular concentration of message. This may be the result of injecting slightly different amounts of RNA or some blastomeres not receiving an equal distribution of message. Problems with leakage from the site of injection may prevent a dose-responsive effect being observed. In any case, the % of embryos that survive after tailbud stage is very low for the embryos injected with high concentrations of message encoding full-length protein and so the % of embryos having later developmental defects is based on a much lower number of embryos, compared to those injected at a much lower concentration of message. These anomalous discrepancies may therefore give a false impression of the relationship between concentration of message and abnormalities.

13.2.3 Analysis of stage 13 embryos, which were bilaterally injected at the 2-cell stage with x-rel, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T.

Injection of x-rel messages does not affect whether cleavage occurs normally. As shown in table 6, abnormal

Table 7.

Summary of scored embryos, showing defects occurring at the tailbud stage, after injection of x-relpSP64T and x-relΔ29pSP64T messages at the 2-cell stage.

Message from the pSP64T constructs was injected at the concentration indicated. Numbers recorded are based on pooled experiments.

* rep. slit anus resulting from slit blastopore.

RNA sample	No. of expts.	No. injected	No. reaching 'tailbud'		Normal		Spina bifida		Reduced head and curl		No head reduced axia		Little axia visible externally	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
			out of those reaching tailbud											
500ug/ml x-relSP64T	2	49	1	2	0	0	0	0	1	100	0	0	0	0
250ug/ml x-relSP64T	5	108	13	12	4	31	1	8	1	8	1	8	6	46
100ug/ml x-relSP64T	6	123	73	59	15*	21	0	0	28	38	15	22	14	19
50ug/ml x-relSP64T	3	66	54	82	28*	21	0	0	21	39	2	4	3	6
500ug/ml uncapped x-relSP64T	4	79	67	85	44	66	19	28	3	4	1	1	0	0
100ug/ml uncapped x-relSP64T	2	45	29	64	16	55	10	34	3	10	0	0	0	0
100ug/ml enticience x-relSP64T	2	41	40	98	33	83	5	13	1	3	0	0	1	3
500ug/ml x-rel 29pSP64T	5	103	56	53	3	5	1	2	28	51	10	18	13	23
250ug/ml x-rel 29pSP64T	3	63	37	59	2	5	1	3	12	32	8	22	14	38
100ug/ml x-rel 29pSP64T	2	49	33	67	2	6	3	9	20	61	8	24	0	0
50ug/ml x-rel 29pSP64T	1	26	16	64	12	75	2	13	0	0	2	13	0	0
500ug/ml uncapped x-rel 29pSP64T	5	90	77	86	66	86	11	14	0	0	0	0	0	0

Figure 41.
Analysis of stage 10-13 embryos injected with x-rel message at the two cell stage.

a) 500ug/ml x-rel uncapped message

Stage 13 embryo (late gastrula); injection of uncapped x-rel message has not affected the timing or normality of gastrulation.

b) 500ug/ml x-rel capped message

This embryo was injected, at the same time as the one described above, with capped x-rel message (prepared from the same DNA sample and under the same conditions). In this case, gastrulation has been delayed and the embryo has only reached stage 10½. The blastopore has not closed, the white region corresponds to mesodermal and endodermal vegetal cells that has not yet been covered by epidermis. At this concentration, the most extreme cases included embryos where gastrulation was arrested.

c) 250ug/ml x-rel Δ221 capped message

Injection of the x-rel Δ221 capped message (which lacks the putative activation region of x-rel) resulted in embryos that seemed to have gastrulated over-vigorously in the final stages - almost the opposite effect of the phenotype seen above. Gastrulation was never delayed as a result of the injection of x-rel Δ221 capped message.

d) 250ug/ml x-rel Δ221 capped message

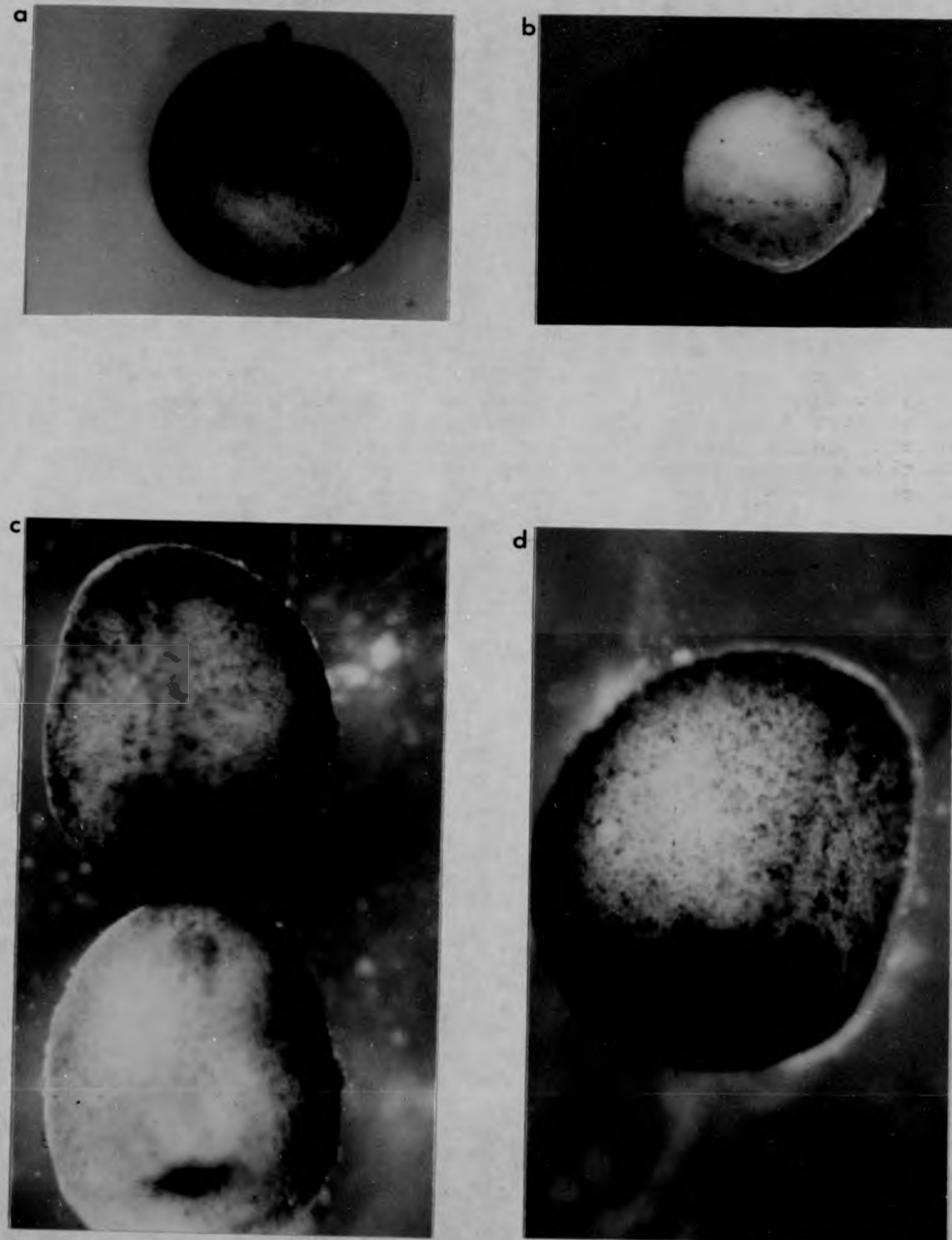


Figure 41.

Analysis of stage 10-13 embryos injected with x-rel message at the two cell stage.

a) 500ug/ml x-rel uncapped message

Stage 13 embryo (late gastrula); injection of uncapped x-rel message has not affected the timing or normality of gastrulation.

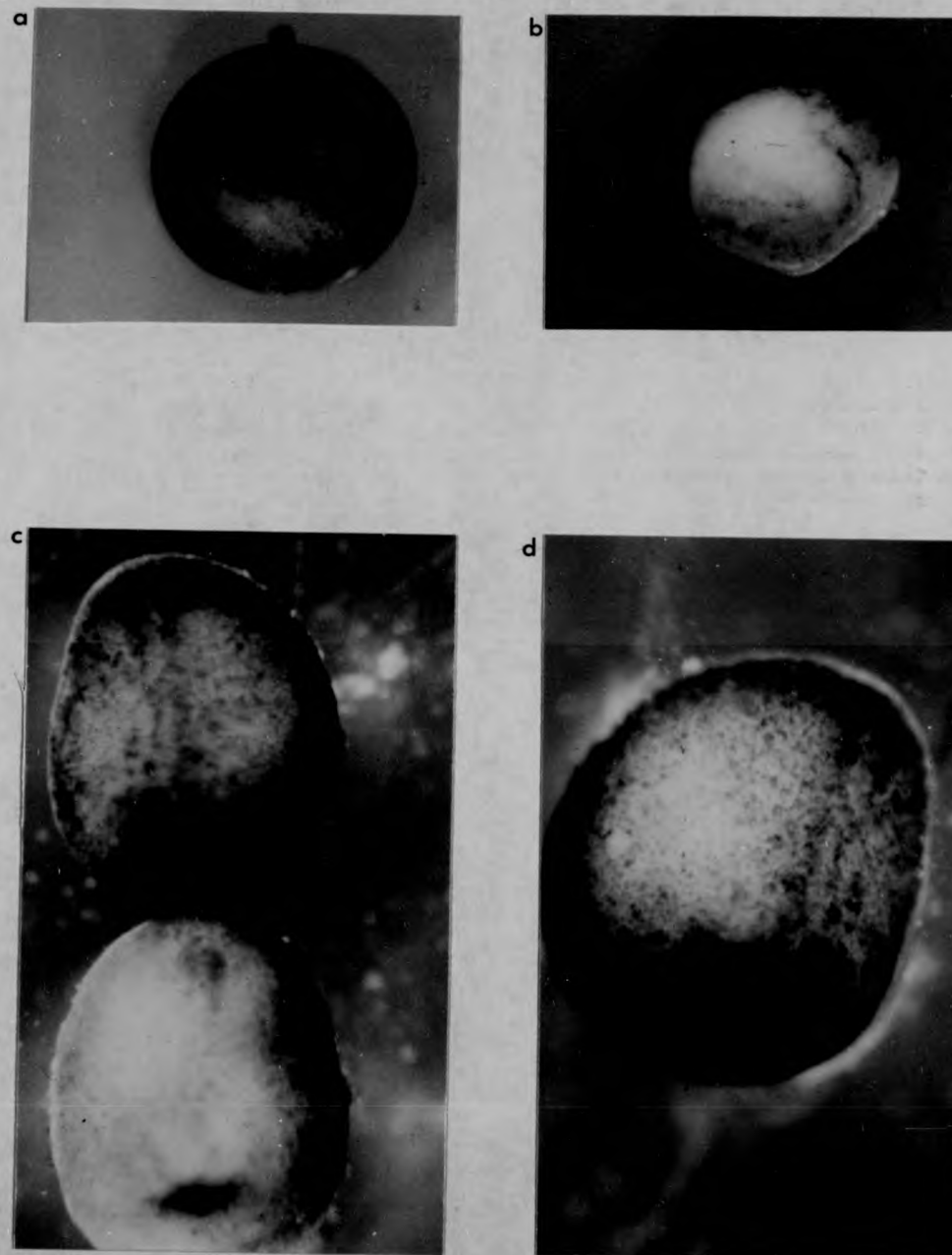
b) 500ug/ml x-rel capped message

This embryo was injected, at the same time as the one described above, with capped x-rel message (prepared from the same DNA sample and under the same conditions). In this case, gastrulation has been delayed and the embryo has only reached stage 10½. The blastopore has not closed, the white region corresponds to mesodermal and endodermal vegetal cells that has not yet been covered by epidermis. At this concentration, the most extreme cases included embryos where gastrulation was arrested.

c) 250ug/ml x-rel Δ221 capped message

Injection of the x-rel Δ221 capped message (which lacks the putative activation region of x-rel) resulted in embryos that seemed to have gastrulated over-vigorously in the final stages - almost the opposite effect of the phenotype seen above. Gastrulation was never delayed as a result of the injection of x-rel Δ221 capped message.

d) 250ug/ml x-rel Δ221 capped message



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cleavage occurs to the same extent in embryos injected with capped x-relpSP64T, x-rel Δ29pSP64T and x-rel Δ221pSP64T message as uncapped messages produced from these constructs. However, at stage 10-10½, there is a direct relationship between the concentration of x-relpSP64T and x-rel Δ29pSP64T injected and the blocking of gastrulation (see table 6). Injection of 20nl of 500ug/ml of x-relpSP64T resulted in a 98% block in gastrulation, whereas injection of 500ug/ml x-rel Δ29pSP64T resulted in a 21% block in gastrulation (in some experiments the % was very high, in others very low; this may reflect the variability in the preparation of the message discussed previously). Even at 500ug/ml, injection of x-rel Δ221pSP64T did not result in the blocking of gastrulation. Figure 41 shows the aberrations in development observed at stage 13 as a result of injection of capped x-relpSP64T and x-rel Δ221pSP64T messages. Injection of uncapped x-relpSP64T message produces perfectly normal embryos (figure 41a), whereas embryos of the equivalent age that have been injected with capped x-relpSP64T message actually resemble normal stage 10-10½ embryos (figure 41b). Up to this stage these embryos show no deviation from controls, but they remain at early stage 10 until controls are late neurulae, when they die. Excess exogenous x-relpSP64T message has caused a block in gastrulation; the white region represents cells that have started to invaginate but are exposed where the blastopore has failed to close properly and the gastrulation movements

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have ceased. Injection of x-rel Δ 22lpSP64T message causes an almost opposite phenotype compared to injection of x-rel Δ 29pSP64T message and x-rel Δ 22lpSP64T message. Gastrulation, rather than being blocked, seems to have occurred overvigorously, at least in posterior regions, marked by the appearance of a pit at the blastopore. Injection of x-rel Δ 22lpSP64T message seemed to more seriously disrupt development, particularly of the head and tail region, at a later stage than gastrulation; the anus remained larger than in controls, leading to a loss of gut material at stage 40 (see later in figure 53a).

Lower concentrations of x-relpSP64T, when injected into embryos, tended to cause a delay in gastrulation. This delay does not indicate that later developmental defects observed with these embryos are simply a result of a termination of movement of mesodermal tissue, since movement does occur and seems to go to completion. Furthermore, if this were the case, the embryos would be more likely to resemble embryos in which gastrulation has been arrested (eg. UV-treated embryos), which lack dorsoanterior structures including heads. Analysis of later stage embryos shows that this characteristic is also apparent in some embryos that were injected with x-rel Δ 22lpSP64T message, which are not in fact delayed at gastrulation.

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13.2.4 Analysis of stage 30-33 embryos, which were injected bilaterally at the 2-cell stage with x-rel, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T.

After about stage 30, it becomes very evident that x-relpSP64T and x-rel Δ 29pSP64T-injected embryos have serious defects in the development of their axis. Only embryos injected with low concentrations (100ug/ml) of x-relpSP64T message were able to survive until this stage, whereas injection of higher concentrations (500ug/ml) of x-rel Δ 29pSP64T message still allowed a reasonable survival rate for some preparations of message, although others caused the same effect as the wild-type. In the most extreme cases, these embryos show very little sign of axis externally, and yet the head and dorsal axis may have formed to some extent, as indicated by the presence of a sucker in some cases (figure 42a) and especially of melanocytes marking the dorsal axis (see later in figure 48c).

Vegetal pole injections of x-rel message, made at the 2-cell stage, appeared to cause less disruption of the axis than the animal pole injections. Figure 43a shows some stage 30 embryos that have been injected, at the vegetal pole with 100ug/ml x-relpSP64T capped message. The axial lengths of the embryos is not reduced as much as in embryos that have been injected with this concentration of x-relpSP64T message in the animal pole (figure 42). The embryos shown in figure 43a seem to have defects in their

Figure 42.

Analysis of stage 30-33 embryos injected with full-length x-rel message at the 2-cell stage.

a) 100ug/ml x-rel capped message

The control embryos for these embryos, which were injected with 100ug/ml capped x-rel message, had reached stage 33. One of these embryos (bottom middle) appeared almost normal. However, the majority show a serious disruption of their mid-axis, resulting in their middle regions being shortened. In most cases their heads are still present, but reduced in size; the most affected ones lack eyes. Their tails, although mostly greatly reduced in length, have formed to some extent.

b) Higher Power of some embryos from a).

A closer inspection of these embryos demonstrates that the head and tail structures are not as much affected as the mid-axis. The darkened areas that appear in a mid-posterior region in the embryo, especially obvious in the mid-ventral region of the central, curved embryo, are indicative of epidermal thickenings.



Figure 43.

Analysis of the equivalent to stage 30-33 embryos injected with full-length x-rel message cont'd.

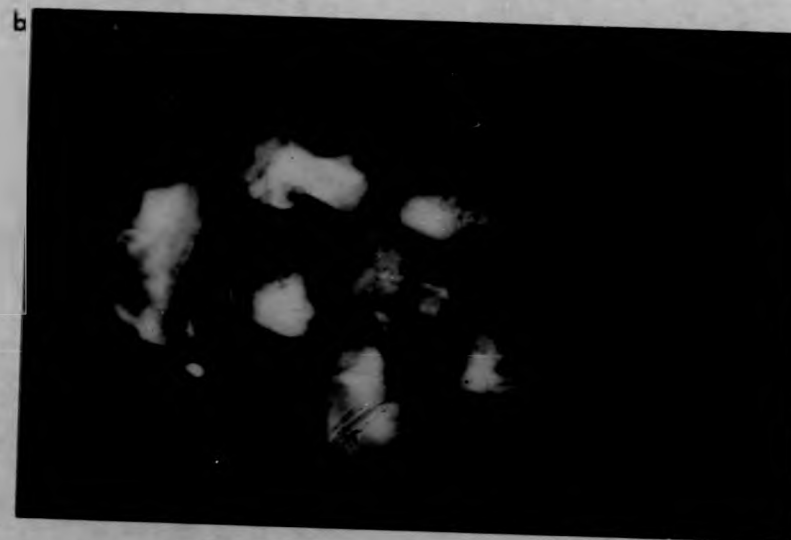
These two sets of embryos demonstrate the variability of the phenotype observed after injection with x-rel message. The embryos were injected with different concentrations of the same batch of synthetic RNA, but in the case of a) the message was injected into the vegetal pole, rather than the animal pole (as was the case for b) and most other injected embryos referred to.

a) 100ug/ml x-rel capped message (injected into the vegetal pole at the two cell stage).

These embryos represent the least extreme phenotype observed after injection of this concentration of message for any of the x-rel clones. Some of these embryos have anterior heads and a couple are missing eyes, but the axial length of these embryos is not as seriously disrupted as observed for animal pole injections. Injection into the vegetal pole may have resulted in the message not being distributed to the regions of the embryo where it would cause most disruption.

b) 50ug/ml x-rel capped message (injected into the animal pole) at the two cell stage.

These embryos represent the most extreme viable phenotype observed after injection of such a low concentration of x-rel message. The whole body axis is completely distorted and the head and tail structures are severely affected. Epidermal thickenings, represented by black areas, (referred to in figure 2) are much more obvious in these embryos.



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anterior structures; some have reduced anterior heads and a couple lack eyes, but most have a normal tail. Vegetal pole injections presumably have a less severe effect because the injected RNA does not reach tissues where it is active, in the same quantities as when animal pole injections are carried out. Animal pole cells form ectoderm and mesoderm tissue, whereas vegetal pole cells form endodermal and mesoderm tissue.

Stage 30 embryos that had been injected with x-rel $\Delta 29$ pSP64T message (figure 44) appear very similar to those injected with the wild-type message, suggesting that the 29 C-terminal amino acids that are deleted probably do not alter the nuclear localisation of x-rel protein or alter its function in a major way. Although a lower percentage of embryos that had been injected with x-rel $\Delta 29$ pSP64T message rather than x-relpSP64T message were blocked at gastrulation, which was probably a message preparation dependent effect (see above), the disruption in later development of the embryos that did gastrulate was very similar to those embryos that had been injected with wild-type x-rel message and was not dependent on preparation of the message. As observed with stage 30 embryos that had been injected with 500ug/ml x-rel $\Delta 29$ pSP64T message (figure 44a), lower concentrations caused a disruption of normal axis development, particularly in the mid-region (data not shown).

Figure 44.

Analysis of the equivalent to stage 30-40 embryos injected with x-rel Δ 29 message.

a) 500ug/ml x-rel Δ 29 capped message

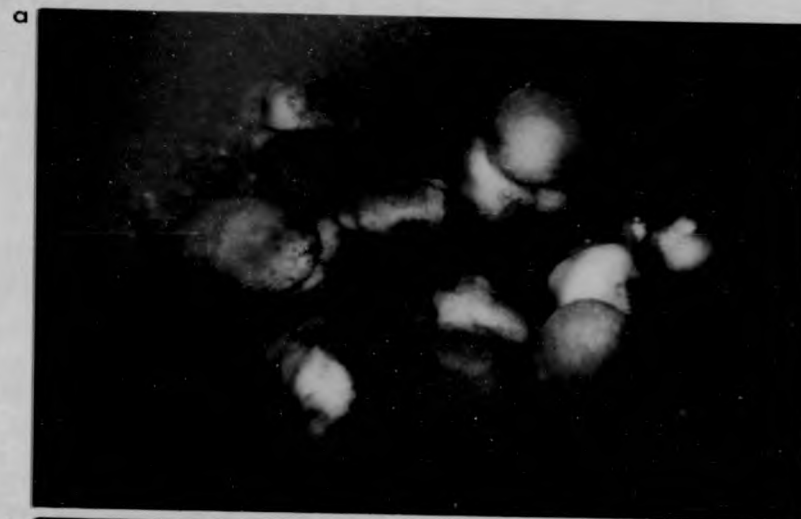
As expected for this high concentration of message, these embryos are completely lacking any recognisable form. They show a very similar phenotype to embryos that have been injected with x-rel message. This case is particularly severe; no head or tail structures can be recognised in most of them.

b) 500ug/ml x-rel Δ 29 uncapped message.

These control embryos are at about stage 31. Nearly all of them have developed completely normally.

c) 250ug/ml x-rel Δ 29 capped message - injected into the vegetal pole.

As described previously, injection of synthetic x-rel messages into the vegetal pole of embryos seems to result in a much less severe phenotype. These injections still produce a variable phenotype; the embryo shown at the top has a distorted mid axis, but the head and tail are not affected to such an extent. The bottom embryo appears normal for an embryo of this age.



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Analysis of stage 30 embryos that had been injected with x-relΔ22lpSP64T message showed an almost opposite phenotype to that observed with embryos that had been injected with x-rel and x-relΔ29pSP64T messages. In making this clone, as described earlier, the aim was to generate a 'loss of function' dominant negative mutant.

There is generally a higher survival rate of embryos injected with x-relΔ22lpSP64T; even at 500ug/ml, a high percentage of embryos survive until tailbud stage. Furthermore, high concentrations of x-relΔ22lpSP64T message tended to produce abnormalities that were more apparent later in development.

Figure 45 shows some stage 30 embryos that had been injected with high concentrations (at or above 250ug/ml) of x-relΔ22lpSP64T message. These embryos have obvious defects in their tail, but the head defects are relatively minor. Most of these embryos have eyes and a sucker. In the most extreme cases (bottom row of figure 45c), the head and tail are missing completely. Generally, injection of high concentrations of x-relΔ22lpSP64T message causes those embryos which have defective anterior structures also to have defective posterior structures, whereas embryos with defective posterior structures are sometimes normal at the anterior end of the embryo (see table 8). Injection of low concentrations of x-relΔ22lpSP64T message (at or below 100ug/ml) produced embryos with a fairly normal axis, but a slightly reduced head and a very reduced tail (see



Figure 45.

Analysis of the equivalent to stage 31 embryos that were injected with x-rel Δ 221 message.

a) 500ug/ml x-rel Δ 221 capped message.

At this high concentration of x-rel Δ 221 message, the embryos are disrupted but the major aberrations of development involve the head and tail regions, rather than the mid axis. The head region is disrupted to some extent, although eyes and a sucker are present, but there is almost a complete lack of any tail structures. The posterior ends of the embryos have a slit-like appearance, where the blastopore has failed to close properly.

b) 500ug/ml x-rel Δ 221 capped message.

This higher magnification of one of the embryos shown above (bottom left) shows the tail to be the most abnormally affected structure. The head is relatively normal. There are folds of skin just behind the head which might be caused by the failure of the dorsal axis to elongate.

c) 500ug/ml x-rel Δ 221 capped message.

Injection of x-rel Δ 221 capped message, like the other x-rel messages (particularly at high concentrations) often produced a high variability of phenotype. The most extreme embryos (shown at the top) lack head and tail structures; the lesser extreme have reduced heads but still lack tails; and the most affected structure of the least extreme cases (shown at the bottom) is again the tail in one case, although in the other it is anterior head.



Figure 46.

Analysis of the equivalent to stage 31 embryos that were injected with x-rel Δ 221 message cont'd.

a) 100ug/ml x-rel Δ 221 capped message.

At this lower concentration of x-rel Δ 221 capped message, most of the embryos appear only slightly abnormal (compared with those injected with higher concentrations of this message). The main defects are a slight reduction in head and a greater reduction in tail size. The mid-axis is, in most cases, normal and straight.

b) 100ug/ml x-rel Δ 221 capped message.

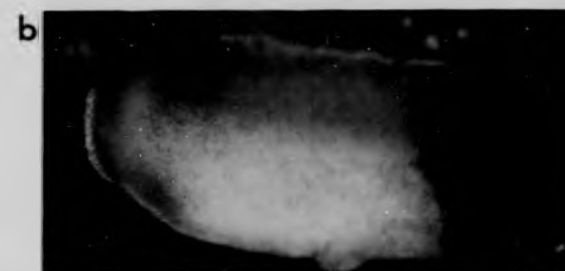
A closer look at one of the embryos from a) shows that the tail is the most affected structure. The head is not seriously disrupted, but it looks more disrupted than it actually is due to the oblique angle of the head with respect to the main body, when the photograph was taken.

c) 100ug/ml x-rel Δ 221 capped message.

This represents a typical example of an embryo injected with x-rel Δ 221 message at this concentration. The embryo appears almost normal; the sucker is more prominent than is the case for higher concentrations and there is evidence of normal eye formation. This embryo presents the first signs of a tail (compared to embryos injected with higher concentrations of this message) although it is still very much reduced in size.

d) 500ug/ml x-rel Δ 221 uncapped message.

About 90% of these control embryos are perfectly normal for their age. A comparison of these embryos with those described above illustrates that the most seriously affected structure, after injection of x-rel Δ 221 message, even at low concentrations, at the two cell stage, is the tail.



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figure 46).

13.2.5 Analysis of stage 39-41 embryos, which were bilaterally injected at the 2-cell stage with x-rel, x-rel Δ 29pSP64T and x-rel Δ 22lpSP64T.

Stage 39-41 embryos that had been injected with 500ug/ml x-relSP64T and x-rel Δ 29pSP64T message, where they survived at all, appeared totally abnormal (figure 47). The mid axis, however, was the most disrupted region of the embryos; a sucker is present in some of these embryos, suggesting that anterior development is disrupted to a lesser extent. At lower concentrations (250ug/ml) of x-relSP64T message (figure 47c), there are some embryos with eyes and suckers, and a small protrusion of a tail. Furthermore, at these lower concentrations of x-relSP64T message (as indicated in table 7), there is less evidence of disruption to the mid axis. However, as demonstrated for all of these phenotypes, there is a consistent variation in the extent to which the embryos are affected. Even at low concentrations such as 100ug/ml, injection of x-relSP64T into embryos caused them to appear at stage 39-40 as though they lacked an axis, and in the most severe cases, the head and tail structures were reduced quite substantially (figure 48). A further example of the variation observed between different experiments, after injection of 50ug/ml x-relSP64T message is shown in figure 49. Figure 49a shows an embryo with a very disrupted axis and signs of epidermal

Figure 47.

Analysis of the equivalent to stage 39-41 embryos that were injected with a range of concentrations of full-length x-rel message.

a) 500ug/ml x-rel capped message.

These embryos are structureless apart from the presence of a small sucker. Again, the mid region of the body is shortened and disrupted due to the aberrations of the mid axis.

b) 500ug/ml x-rel uncapped message.

About half of these control embryos are completely normal for stage 40 embryos (shown on the right). The other half of the embryos resemble 'spina bifida' (shown on the left), which is a common problem with injected embryos and can occur even in embryos that have not been injected.

c) 250ug/ml x-rel capped message.

Even at half the concentration of x-rel message that produced the embryos described in a), these embryos appear much more normal. The mid-axis is still severely disrupted, but the head is significantly more developed in most, as noted by the appearance of eyes and a sucker. There is also evidence of a tail analage in most.

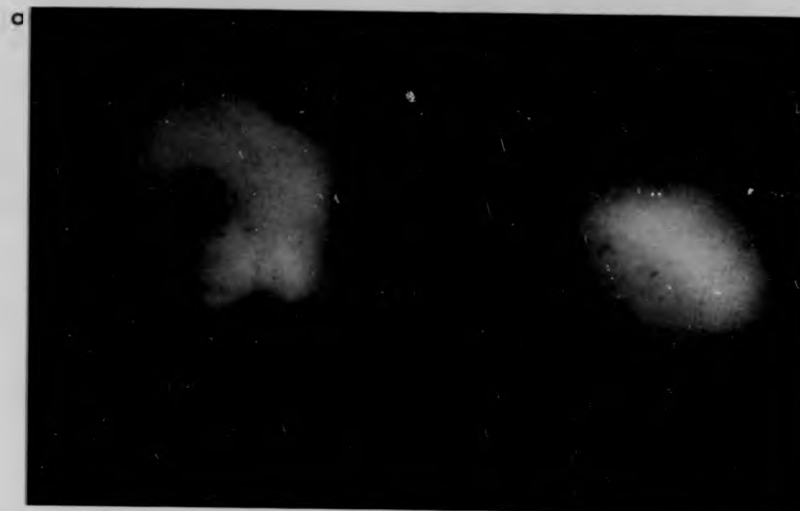


Figure 48.

Analysis of the equivalent to stage 39-41 embryos that were injected with a range of concentrations of x-rel message cont'd.

a) 100ug/ml x-rel capped message.

At this lower concentration, there is a greater proportion of embryos whose mid axis is not quite so disrupted. However, some of these embryos are as grossly affected as those injected with a higher concentration of x-rel message. This variable range of phenotypes for each concentration of message injected was a consistent observation, except for the very highest concentration of most RNA preparations.

b) 100ug/ml x-rel capped message.

This embryo is an example of a less extreme case. The head and tail are again the least affected regions, but the mid region of the embryo is disturbed due to disruption of the mid axis.

c) 100ug/ml x-rel capped message.

This embryo presents the most extreme case at this concentration. The head and tail are completely absent and the mid-axis is extremely disrupted, but its presence is outlined by the melanocytes.

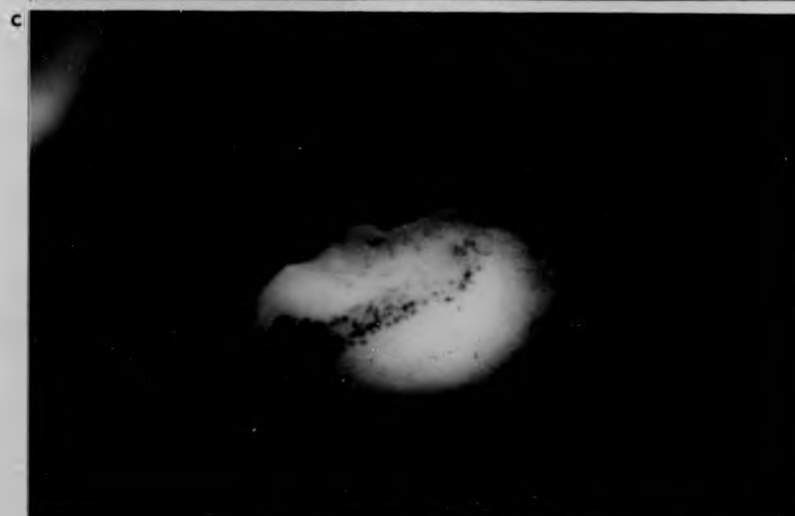
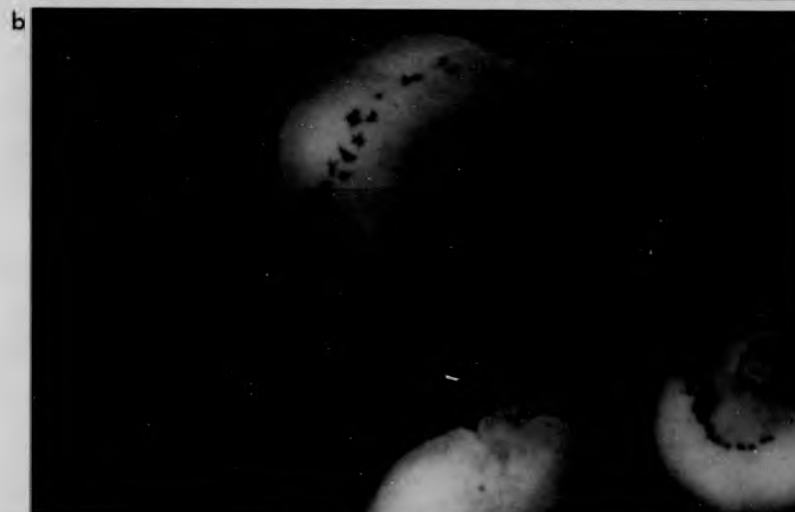
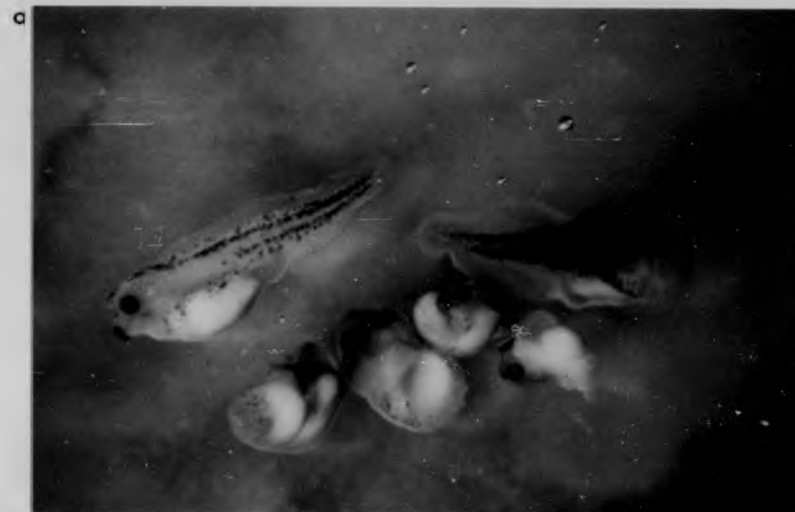


Figure 49.

Analysis of the equivalent to stage 39-41 embryos that were injected with full-length x-rel message.

a) 50ug/ml x-rel capped message.

Even at this low concentration of injected x-rel message, some embryos were very malformed. This particular embryo illustrates the epidermal thickenings (represented by the dark patch just below the anterior region) that were commonly observed for embryos injected with x-relSP64T synthetic capped transcripts.

b) 50ug/ml x-rel capped message.

In general, injection of such a low concentration of x-rel message resulted in embryos that appeared completely normal for stage 40.



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thickenings, which were extremely common in embryos that were injected with a lower concentration of x-relSP64T message. Figure 49b, however, shows another batch of embryos which appeared perfectly normal after injection with 50ug/ml x-relSP64T message.

The epidermal thickenings referred to above (figure 49a; see also figure 42b) were never apparent in embryos that were injected with x-rel Δ 221pSP64T message. To demonstrate clearly that these structures were, indeed, due to thickening of the epidermis, sections of x-relSP64T message-injected embryos were stained with an antibody to an epidermal antigen. These results are shown in figure 50.

One might predict that since a lower percentage of embryos that had been injected with x-rel Δ 29pSP64T message were delayed at gastrulation, later disruptions in development might not have been quite so affected. This is generally true, but in extreme cases, where the axis has not developed normally, they are indistinguishable from x-relSP64T-injected embryos. Figure 51a shows extreme cases of stage 39 embryos, that had been injected with 500ug/ml x-rel Δ 29pSP64T message, which are lacking head and tail structures and whose axial length is severely reduced; figure 51b shows less extreme cases and figure 52 shows examples where the head and tail structures are not as disrupted as a result of injection of lower concentrations of x-rel Δ 29pSP64T compared to x-relSP64T. The presence of eyes, a sucker and a normal tail is much

Figure 50.

Transverse sections of mid to late 30 stage embryos injected with x-rel messages and stained with an antibody to an epidermal antigen.

Embryos were fixed and sectioned as described in Methods Section 3.29, except that SDS treatment was omitted. Sections were stained by indirect immunofluorescence with a mouse monoclonal antibody (2F7.C7; see Jones and Woodland, 1986) to an epidermal antigen and rhodamine-conjugated rabbit anti-mouse (essentially as described for rabbit polyclonal antibodies and FITC-conjugated goat anti-rabbit; see Methods Section 3.29).

- a) 500ug/ml x-rel uncapped message.
T.S. middle section.
Normal epidermis.
- b) 100ug/ml x-rel capped message.
T.S. region of epidermal thickening.
Epidermal thickening apparent.
- c) 100ug/ml x-rel capped message.
T.S. region of epidermal thickening.
Epidermal thickening apparent, and some epidermis formed within the embryo.

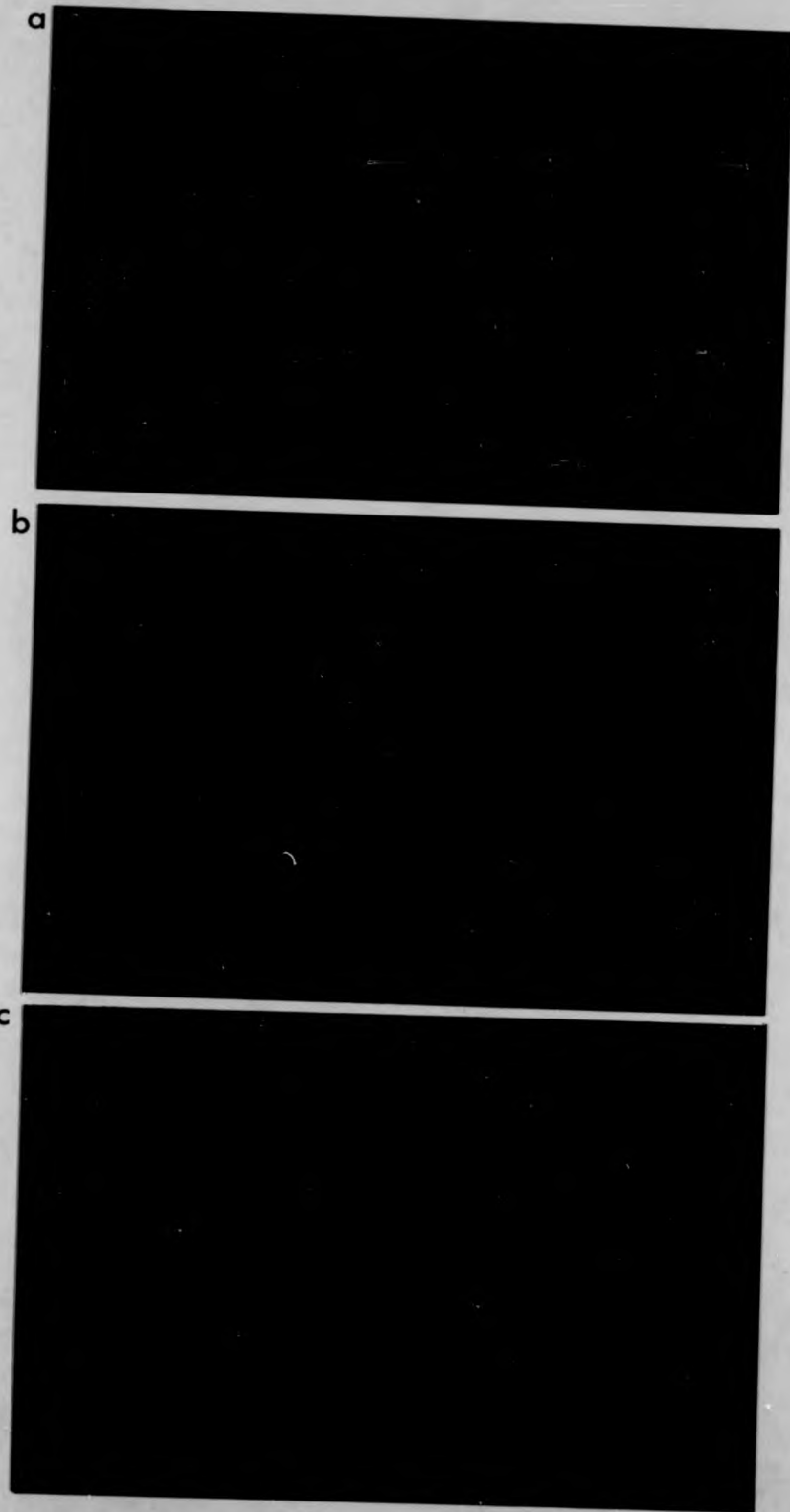


Figure 51.

Analysis of the equivalent to stage 39-41 embryos that were injected with x-rel Δ 29 message.

a) 500ug/ml x-rel Δ 29 capped message.

This is an extreme case of abnormal embryos as a result of injection of x-rel Δ 29 message.

b) 500ug/ml x-rel Δ 29 capped message.

These embryos are a less extreme case of embryos injected with this message at such a high concentration. They are less disrupted than the least extreme examples of those injected with full length, x-rel, message at the equivalent concentration. The aberrations are, again, mainly in the mid axis and the tail is reduced in size and tends to curve upwards. The head is the least affected structure and the eyes and sucker appear to have developed normally.

c) 500ug/ml x-rel Δ 29 uncapped message.

Three of these embryos appear as expected for embryos of this age (about stage 40). The remainder resemble 'spina bifid' embryos, which has not been specifically caused as a result of injection of this message (see earlier). These are controls for the experiments in b).



Figure 52.

Analysis of the equivalent to stage 39-41 embryos that were injected x-rel Δ 29 message cont'd.

a) 250ug/ml x-rel Δ 29 capped message.

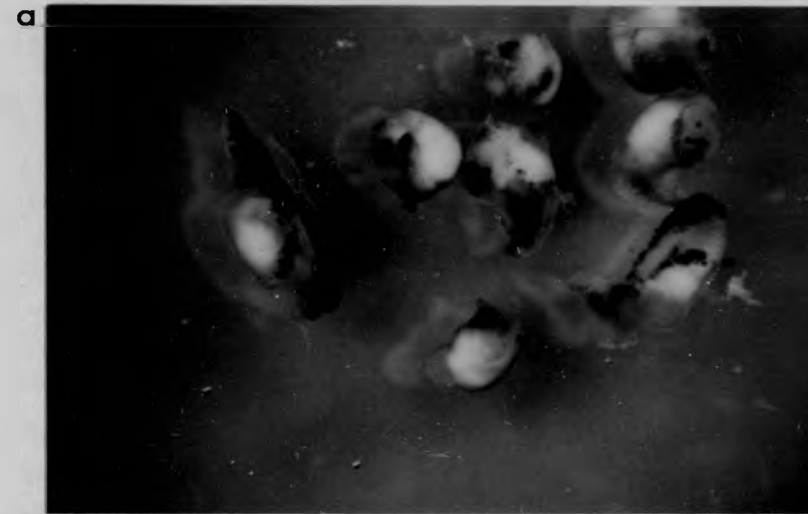
The least extreme examples shown here have reasonably well developed heads in many cases and tails, but the mid-axis is still disrupted to a large extent in most cases.

b) 100ug/ml x-rel Δ 29 capped message.

At this concentration, a greater proportion of embryos have a more complete mid-axis. Some of the least affected embryos appear identical to embryos injected with uncapped x-rel Δ 29 message. In nearly every case, the head has developed to an extent, where eyes and suckers have formed. Most of these embryos also have tail-like structures.

c) 100ug/ml x-rel Δ 29 capped message (vegetal pole).

A closer look at an embryo from b) illustrates that the head region is fairly normal. However, the mid-axis is shortened and the reduced tail has an abnormally upward curve.



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more commonly observed in stage 40 embryos that had been injected with x-rel $\Delta 29pSP64T$ message. Figure 52c shows that injection of x-rel $\Delta 29pSP64T$ message into the vegetal pole, as described for x-rel $pSP64T$ message, results in less extreme disruption; even the most severe embryos developed head and tail structures and some kind of extremely obvious axis. As described for x-rel $pSP64T$, injection of lower concentrations of x-rel $\Delta 29pSP64T$ message have more serious effects later in development; the axis is not as disrupted at lower concentrations but the head is still reduced in size until much lower concentrations (below 100ug/ml).

Stage 40 embryos that had been injected with x-rel $\Delta 221pSP64T$ message (figure 53) at the 2-cell stage, display the same characteristics as those observed at stage 31 (see figure 46). Extreme examples of embryos injected with 500ug/ml x-rel $\Delta 221pSP64T$ message lacked a tail, had a reduced head and shortened axis (figure 53a). Less extreme examples of embryos injected with x-rel $\Delta 29pSP64T$ message are shown in figure 53b. From top to bottom, the embryos become less disrupted; the least extreme embryos have normal axial lengths and their heads are not as reduced in size. However, even the least extremely disrupted embryos lack a tail, or it is reduced.

Figure 53.

Analysis of the equivalent to stage 40 embryos that were injected with x-rel Δ 221 message.

a) 500ug/ml x-rel Δ 221 capped message.

These embryos represent extreme examples of embryos injected with this high concentration of message. Very little tail structure is really apparent in two embryos and its reduced in the third. The head is extremely reduced in size in only one embryo. The eyes and sucker have formed reasonably normally in two of these embryos.

b) 250ug/ml x-rel Δ 221 capped message.

The head of this stage 40 embryo has failed to develop normally; there are areas of this region where eyes and a sucker seem to have begun to develop, but for an embryo of this age these structures should have formed completely. The main body axis is not seriously affected, but the reduced tail has an upward 'kink' to it.

c) 250ug/ml x-rel Δ 221 capped message.

An enlargement of the tail of the embryo shown in b) illustrates the abnormal curved shape of the tail and its almost tube-like tip, which grew through the fin. This was also seen in embryos injected with full-length x-rel message (for example, those shown in figure 2a).

d) 500ug/ml x-rel Δ 221 uncapped message.

These control stage 40 embryos illustrate, in particular, how disrupted tail formation is in the embryos described above.

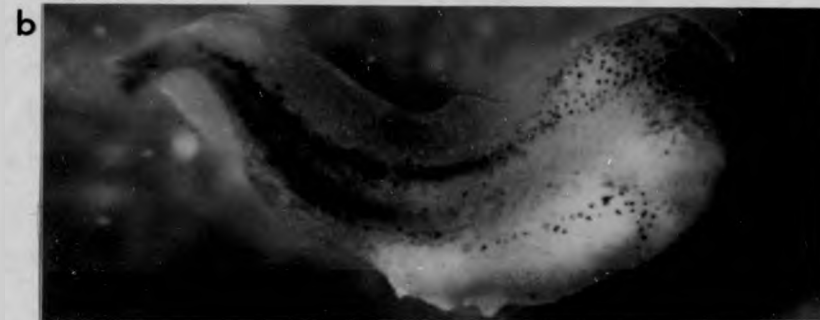
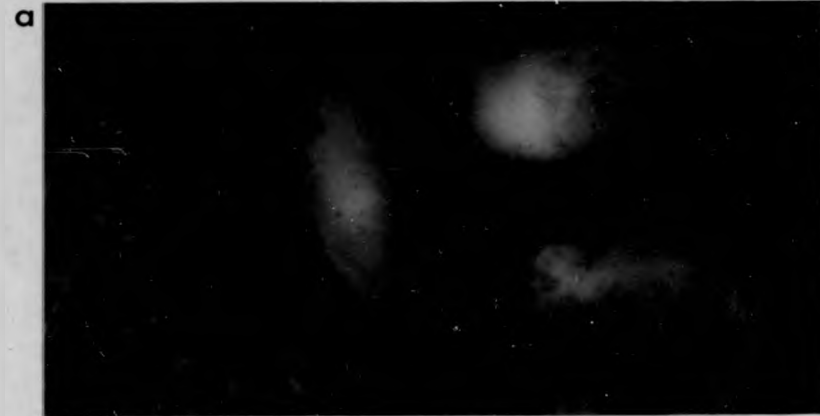


Figure 53.

Analysis of the equivalent to stage 40 embryos that were injected with x-rel Δ 221 message.

a) 500ug/ml x-rel Δ 221 capped message.

These embryos represent extreme examples of embryos injected with this high concentration of message. Very little tail structure is really apparent in two embryos and its reduced in the third. The head is extremely reduced in size in only one embryo. The eyes and sucker have formed reasonably normally in two of these embryos.

b) 250ug/ml x-rel Δ 221 capped message.

The head of this stage 40 embryo has failed to develop normally; there are areas of this region where eyes and a sucker seem to have begun to develop, but for an embryo of this age these structures should have formed completely. The main body axis is not seriously affected, but the reduced tail has an upward 'kink' to it.

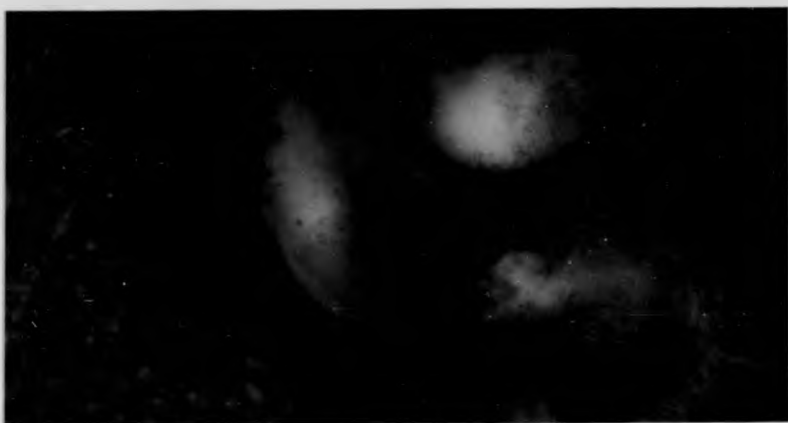
c) 250ug/ml x-rel Δ 221 capped message.

An enlargement of the tail of the embryo shown in b) illustrates the abnormal curved shape of the tail and its almost tube-like tip, which grew through the fin. This was also seen in embryos injected with full-length x-rel message (for example, those shown in figure 2a).

d) 500ug/ml x-rel Δ 221 uncapped message.

These control stage 40 embryos illustrate, in particular, how disrupted tail formation is in the embryos described above.

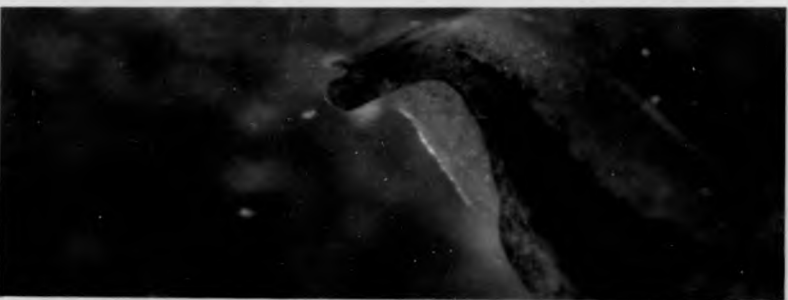
a



b



c



d



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13.3 Histological analysis of the internal organisation of embryos that had been injected with x-relpSP64T, x-rel Δ 29pSP64T and x-rel Δ 22lpSP64T messages.

The observations described above are necessarily impressionistic. In order to confirm and extend them to detailed tissue organisation, the histology of affected embryos was examined. One problem with this analysis is that it was necessary to be somewhat selective in what was examined, but an effort was made to examine representatives of what was described above. In addition to revealing which disrupted tissue type (neural, epidermal or somites for instance) was causing the main defects, this technique would allow a more accurate picture of the abnormalities resulting from injection of x-rel messages. Some embryos, for example, might have appeared normal externally, but upon histological examination could have revealed a more subtle or less extreme disruption of the same nature as described for the external phenotype.

13.3.1 Histological sections of embryos that had been injected with x-relpSP64T and x-rel Δ 29pSP64T messages.

Histological sections of embryos that were only a little abnormal after 2-cell embryos were injected with x-relpSP64T and x-rel Δ 29pSP64T messages, compared to sections of normal embryos, reveal that the most

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disorganised region of the embryos is the middle section, where most tissues are to some degree disorganised and somites are unsegmented. An exception is the notochord, which almost always appeared normal. Furthermore, the dorsoventral distribution of tissues does not seem to be disrupted and the endoderm was never disrupted. The internal order of the head region, in particular, is not too different from that of control embryos. Figure 54 shows that the main structures in the head; eye, brain and so on, appear normal, but compared to the control embryo (figure 54b), the head mesenchyme is slightly disordered. However, since the eyes, representing one of the most anterior positional values, have also formed normally, it would suggest that gastrulation, although delayed as a result of injection of x-relpSP64T and x-relΔ29pSP64T messages, has not been seriously inhibited and prospective anterior mesoderm has reached its normal position. Figure 54c shows a transverse section taken just behind the head, showing tissues (particularly of the somites and CNS) which are more distorted than in the control embryo. The notochord, however, appears to have formed normally.

Transverse sections taken toward the middle of the embryo (figure 55) reveal a greater disturbance of tissue formation. Although all tissue types appear to have acquired their normal differentiated state, the formation of structures such as the notochord, and regions of CNS and muscle have formed in an aberrant way. There are no obvious

Figure 54.

Histological sections of stage 37 embryos.

a) 250ug/ml x-rel capped message.

T.S. head.

Injection of x-rel message does not significantly disrupt the organisation of tissues in the head in moderately affected embryos. The eye has formed normally and the brain ventricle is quite large. The visceral pouches and fore gut appear fairly normal. However, compared to the control section, (shown below) the head mesenchyme is not quite so ordered.

b) 500 ug/ml x-rel uncapped message.

T.S. head.

This is a section through the same region of the head as shown above.

c) 250ug/ml x-rel capped message.

T.S. anterior region just behind the head.

This region is fairly normal except that the nervous system is reduced in size and appears distorted. Formation of the notochord, however, has not been disrupted, although it appears flattened a little. Compared to the control section (shown below), there is slightly less muscle tissue surrounding the notochord and it is fairly disorganised. The organisation of the endodermal cells is not affected to a great extent.

d) 500ug/ml x-rel uncapped message. .

T.S. anterior region.

This control section illustrates the overall better organisation of the nervous system and somites and shows there is not much difference between c) and d) of the endodermal cells.

For all the following photographs of histological sections:
N represents nervous system; Nc represents notochord; S represents somites.

The bar (1cm.) represents 210uM.



Figure 55.

Histological sections cont'd

a) 250ug/ml x-rel capped message.

T.S. middle section of stage 42 embryo.

As sections are made towards the middle of embryos that have been injected with x-rel message, more disruption, particularly of somites and nervous system, is apparent. The notochord is reduced in size and the nervous system is extremely disrupted. The somites lack any kind of organisation. The endodermal cells are not affected to a great extent.

The bar (1cm.) represents 210uM.

b) 250ug/ml x-rel capped message.

T.S. middle section: higher magnification of a).

A closer look at the dorsal region of this section shows that the notochord is reduced in size and illustrates the complete lack of organisation of both the nervous system and the surrounding somites.

The bar (1cm.) represents 85uM.

c) 250ug/ml x-rel capped message.

T.S. middle section of stage 37 embryo.

This section represents an extreme example of disruption in the middle region of an embryo injected with x-rel message. The notochord is reduced in size (not apparent in other sections). The nervous system is reduced to some extent and also quite disrupted. The melanocytes have migrated in a very haphazard fashion and are scattered around the nervous system and somites. The tissues on the ventral side of the embryo are completely disorganised.

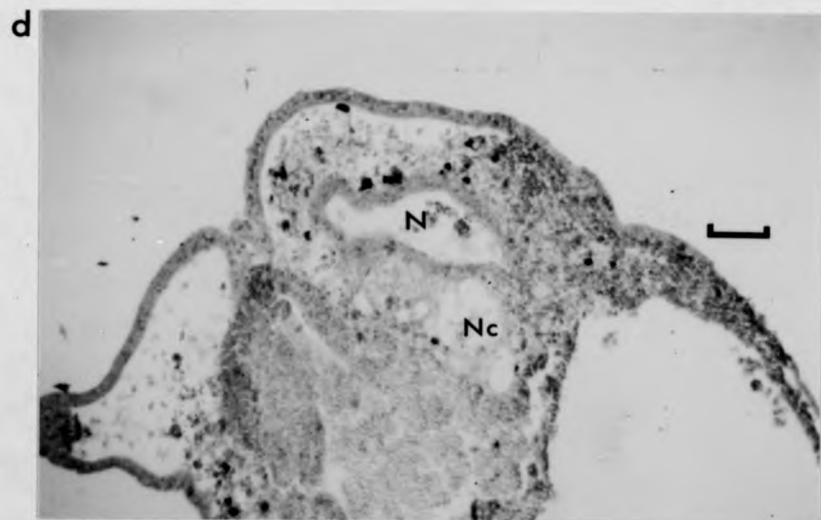
The bar (1cm.) represents 210uM.

d) 250ug/ml x-rel capped message.

T.S. middle section: higher magnification of c).

This photograph highlights the disruption and lack of segmentation of the somites around the notochord, which itself is lacking any organisation and is reduced in size. The lack of proper structure in the central nervous system is especially notable.

The bar (1cm.) represents 85uM.



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defects in the endoderm and non-neural ectoderm, compared to control embryos. There is a roughly similar amount of mesoderm in the middle sections of capped x-relSP64T message-injected embryos and uncapped x-relSP64T message-injected embryos again suggesting that gastrulation has been completed in these embryos. However, the lack of elongation of these embryos and their disorganisation makes detailed quantitation from sections difficult. Between different embryos, there is a variable degree of disorder in the mesoderm and in the neural ectoderm. At high concentrations of x-relSP64T message, the somites may be fused along the mid-line and the CNS is deformed. There is an obvious disturbance in the correct formation of somites. Segmentation of somites from unsegmented mesoderm begins during neurulation, which correlates with the temporal expression of x-rel message at high levels (section 6.2). One can postulate that x-rel function, which may begin before or during gastrulation, may not be important until neurulation and may, at this time, be activating another gene (or genes) that is subsequently directly responsible for the correct formation of somites. XlHbox1, when injected into embryos also produces disruption of somites (Harvey and Melton, 1988). Somites in posterior regions are as disordered as those in anterior regions, even though they form hours after anterior somites, suggesting that a gene or gene(s) whose function is affected by x-rel expression may be active for a long

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period during neurulation.

Figure 56 shows transverse sections through an embryo that had been injected with 250ug/ml capped x-relpSP64T. Towards the posterior end of the embryo, there is a slightly more organised patterning of somites than in the mid-region. However, compared to the control embryo shown in figure 56c and 56d, the blocks of somites are still disorganised and expand into areas where they are not normally present; perhaps staining with fibronectin, which normally outlines the somites, might reveal the extent of their distribution. The neural crest cells also show an abnormal distribution (figure 56a and 56b) compared to control embryos (figure 56d); a large proportion of these cells have failed to migrate away from the nervous system and those that have are scattered in complete disarray. At the posterior end of the embryo, there is slightly less internal disruption of tissues than in the middle region. However, there is still little mesodermal differentiation and only a poorly developed nervous system, although a substantial notochord is usually present (figure 57).

As observed externally, histological sections of embryos that had been injected with x-rel Δ 29pSP64T message show a very similar pattern of disorganisation. Figure 58 shows the middle section is again the most seriously disrupted region. The nervous system is poorly developed and again the somites are completely disrupted. The notochord, however, is reasonably well ordered. X-rel, therefore,

Figure 56.

Histological sections cont'd.

a) 250ug/ml x-rel capped message.

T.S. middle-posterior section of stage 42 embryo.

The same characteristics of the phenotype described above apply to this more posterior section. The somites, however, are perhaps slightly better organised than those shown in the middle section.

The bar (1cm.) represents 210uM.

b) 250ug/ml x-rel capped message.

T.S. middle-posterior section: higher magnification of

a).

This section illustrates that the somites lack organisation, which has caused them to expand into the region normally occupied by neural crest cells, adjacent to the nervous system. Again the melanocytes have failed to migrate to the correct regions and are indiscriminately positioned.

The bar (1cm.) represents 42uM.

c) 500ug/ml x-rel anti-sense message.

T.S. middle-posterior section of stage 42 embryo.

This control section of an embryo injected with anti-sense x-rel message shows the normal organisation of the somites in this region and the presence of a large notochord. The nervous system is well structured and detached from the surrounding somites.

The bar (1cm.) represents 210uM.

d) 500ug/ml x-rel anti-sense message.

T.S. middle-posterior section: higher magnification of

c).

A higher magnification of this section shows more clearly the nervous system and the correct segmentation pattern of the somites surrounding this region and the notochord.

The bar (1cm.) represents 42uM.

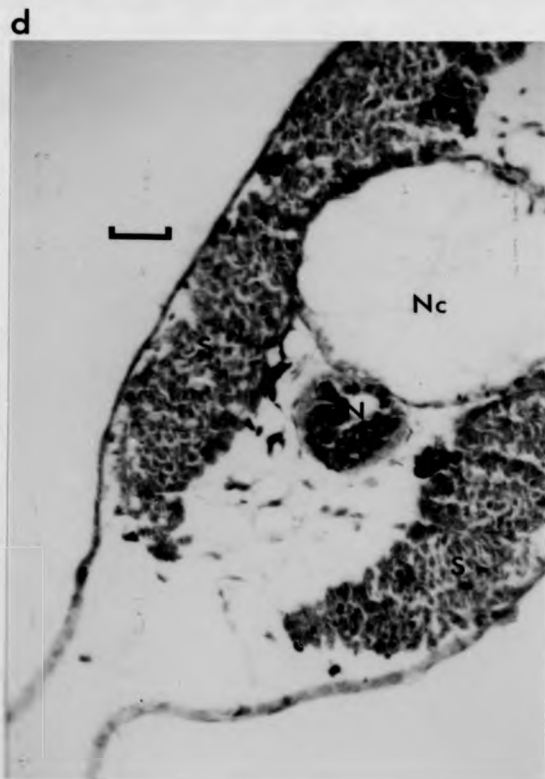
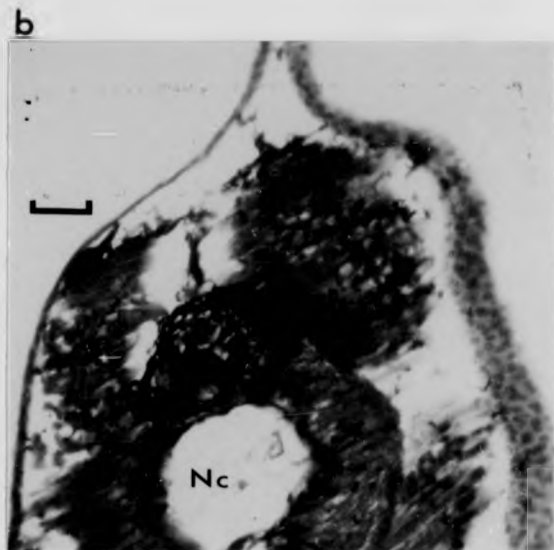


Figure 57.

Histological sections cont'd.

a) 250ug/ml x-rel capped message.

T.S. posterior region of stage 42 embryo.

This section through the posterior region of a embryo injected with x-rel message represents a moderate example of overall abnormal development caused by this message. However, there is still great disruption in the organisation of the somites and endodermal cells. The notochord is quite large and well organised, but the muscles are very abnormal. The nervous system is reduced in size, in a way that is very characteristic of this region after injection of this message.

The bar (1cm.) represents 210uM.

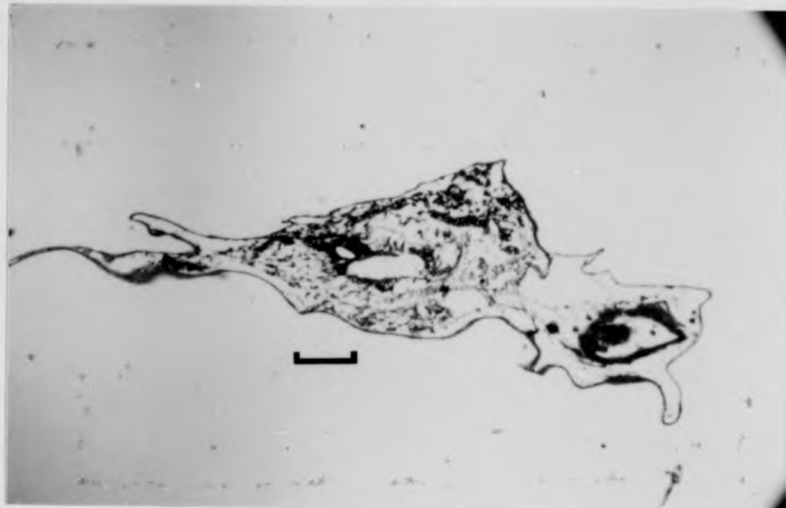
b) 250ug/ml x-rel capped message.

T.S. posterior region: higher magnification of a).

Higher magnification of this section illustrates the complete disruption of somites, and the organisation of the central nervous system is also abnormal and it is greatly reduced in size, although the size of the notochord is not affected.

The bar (1cm.) represents 85uM.

a



b

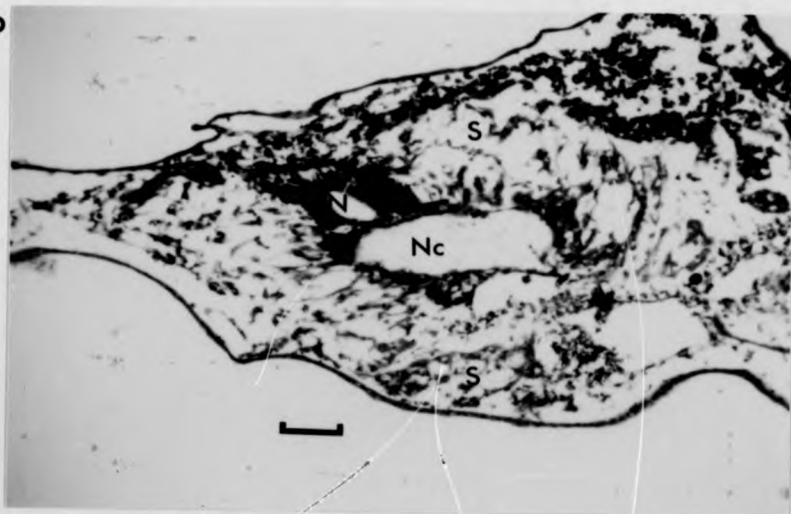


Figure 58.

Histological sections cont'd.

a) 500ug/ml x-rel Δ 29 capped message.

T.S. middle region of stage 37 embryo.

Injection of x-rel Δ 29 capped transcripts into embryos generally produced the same characteristics of phenotype as the full-length x-rel message. This section shows complete disorganisation of the somites in the mid-posterior region. There is a poor nervous system in which the tissue distribution is also very disrupted. As was the case for the embryos injected with x-rel message, the notochord is fairly normal.

The bar (1cm.) represents 85uM.

b) 250ug/ml x-rel Δ 29 capped message.

T.S. middle region of stage 37 embryo.

At this lower concentration of message, there is still disorganisation of the somites, a greatly reduced and poorly developed nervous system and even the endodermal cells are disorganised. However, the notochord is again relatively normal, both in size and structure.

The bar (1cm.) represents 85uM.

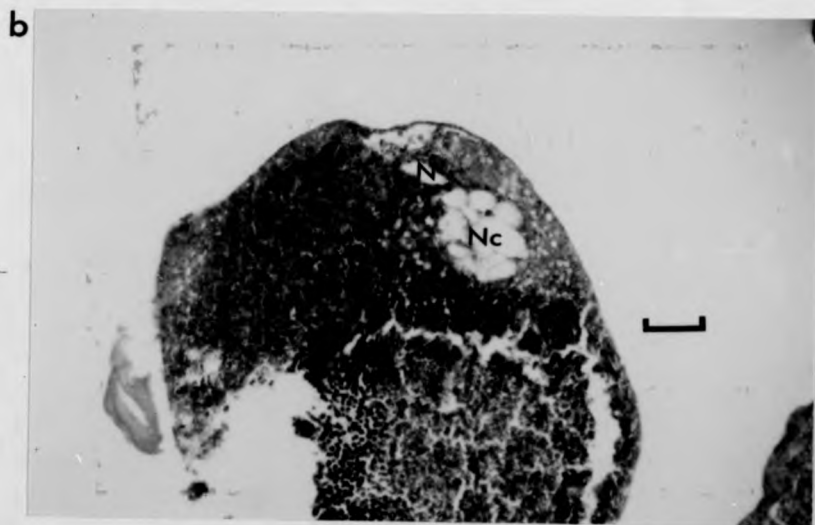
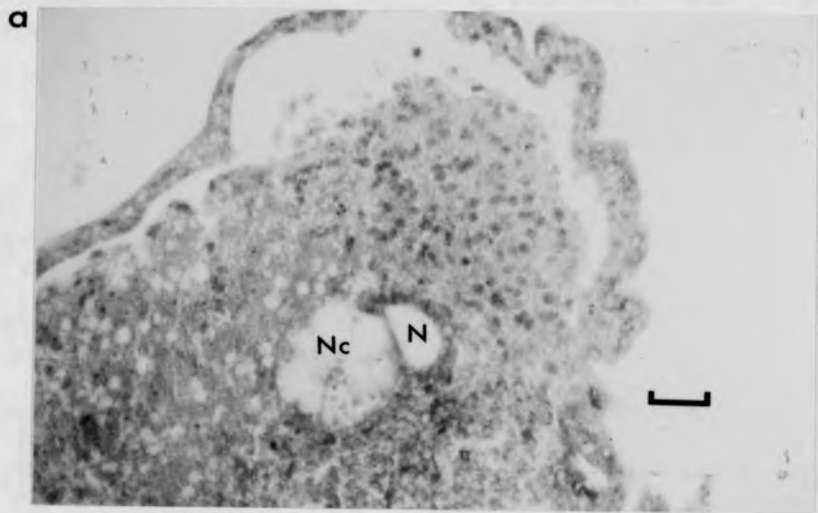


Figure 59.

Histological sections cont'd.

a) 500ug/ml x-rel Δ 221 capped message.

T.S. head region.

This section shows a large amount of disorganisation of all the tissues in the head region. The eye has developed reasonably well, but the size of the head itself is greatly reduced. The organisation of the endodermal cells is not badly affected.

The bar (1cm.) represents 85uM.

b) 500ug/ml x-rel Δ 221 capped message.

T.S. head region.

This section shows the complete disorganisation of the nervous system. The epidermis appears to be thickened in the head region.

The bar (1cm.) represents 85uM.

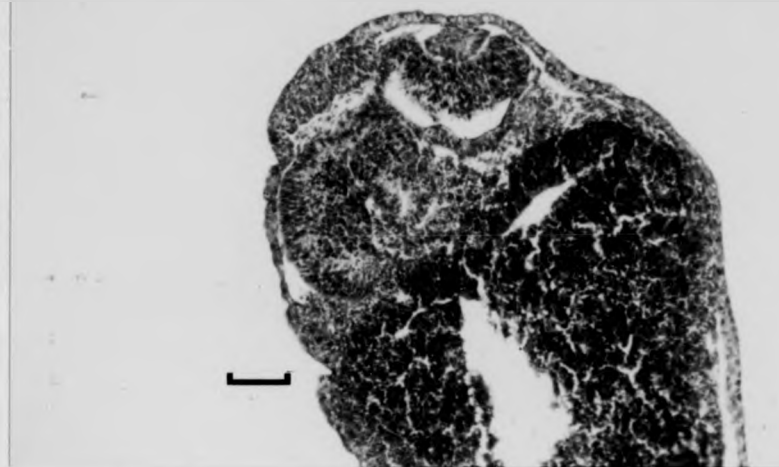
c) 500ug/ml x-rel Δ 221 capped message.

Horizontal middle region.

This section indicates that the tissues in the middle region of the embryo are less disrupted than the anterior regions. There are about the correct number of somites and they are fairly well segmented; there is some unsegmented somitic mesoderm. The central nervous system is about the correct size, but appears elongated. The notochord is about the normal size and is fairly well ordered.

The bar (1cm.) represents 85uM.

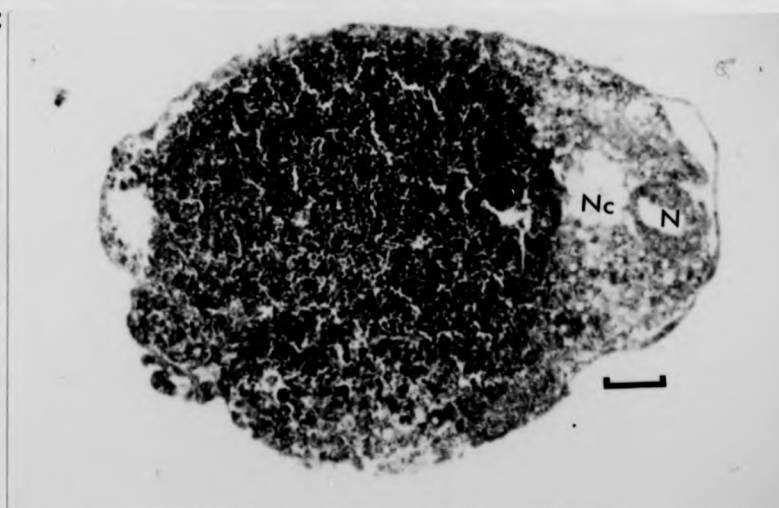
a



b



c



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seems to disrupt certain structures that have developed from mesoderm (eg. somites) and yet hardly disrupt other mesodermally-derived structures such as the notochord. Structures derived from the neuroectoderm are also disrupted and there seems to be more disruption to tissues on the dorsal side (with the exception of the notochord), compared to the ventral side of the embryo. This may reflect the greater ease with which one can discern the organisation in this region, which in any case shows more complexity than ventral tissues at this stage.

13.3.2 Histological sections of embryos that had been injected with x-rel 221pSP64T message.

Histological sections of embryos that had been injected with x-rel 221pSP64T message show, as inferred from external observation, that development of the head and tail are the most seriously disrupted regions of the embryo. Figure 59 presents sections taken from the anterior end to the posterior end of a seriously affected embryo, illustrating the position-dependent disruption of internal tissues as a result of injection of x-rel 221pSP64T message. The anterior sections, in particular, are extremely disrupted. The eye has developed reasonably well (figure 59a), which might indicate that the effect is reasonably specific. The head tissues have reached, at least to a superficial examination, their normal differentiated state, but lack a great deal of

Figure 59 cont'd.

d) 250ug/ml x-rel Δ 221 capped message.
T.S. posterior region.

The spinal cord is reduced in size - the central canal is much larger than it should be. The endodermal yolk mass appears fairly normal. The main difference in abnormalities between a posterior section and a middle section (shown above) is that the organisation of tissues located dorsally, especially somites, is much more disrupted in the middle section.

The bar (1cm.) represents 85uM.

e) 250ug/ml x-rel Δ 221 capped message.
T.S. posterior region.

This section is a more posterior one of the same embryo shown in d).

The bar (1cm.) represents 85uM.



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organisation, especially in the CNS, which is greatly disrupted. The internal organisation of the middle region, in contrast to embryos that had been injected with x-relSP64T and x-rel Δ 29pSP64T message, shows least disruption. The notochord (figure 59c) is large and well organised; there are roughly the correct number of somites. As sections are made closer to the posterior end of the embryo, disruption changes from one tissue to another. Figure 59d shows that the main disruptions occurring in the posterior end are of the CNS, rather than of the somites. This disruption to posterior regions of somites bordering the tail was particularly obvious in whole mounts stained with a muscle-specific monoclonal antibody (not shown).

13.4 Conclusions from external and internal observations of embryos that had been injected with x-relSP64T, x-rel Δ 29pSP64T and x-rel Δ 221pSP64T messages.

13.4.1 Summary and interpretation of data.

Injection of x-rel messages into embryos produces embryos with a different degree of disorganisation in some regions, but a consistent lack of any effect in certain other regions. For example, the dorsoventral distribution of tissues is always preserved, the nervous system and somites are always present, the notochord is not disorganised to any great extent, melanocytes are usually present and the endoderm is always well organised.

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The main conclusion from external observations of phenotype from embryos injected with x-relSP64T and x-relΔ29pSP64T is that overexpression of these transcripts leads to serious disruption in the middle of the embryo and in the formation of the central body axis. Least disruption was observed for the head and the tail region. These regions may be interpreted as the regions where x-rel is normally most abundant because in these regions x-rel activity may already be close to saturation. This case has been argued for Xhox3, which is maximally expressed in the posterior mesoderm and injection of Xhox3 results in anterior deficiencies (Ruiz i Altaba and Melton, 1989c). However, it could be envisaged that the opposite is true, for example, x-rel may require ancillary factors for its function which are only present where it is normally expressed. It is therefore possible to formulate a hypothesis that x-rel is most highly expressed in the head and tail and is possibly not expressed to a significant amount in the middle region of the embryo.

X-rel overexpression most effectively disrupts the organisation of mesoderm and neuroectoderm in the mid axis. One possibility is that x-rel may be disrupting cell adhesion. Epidermal thickenings were a common feature of embryos injected with x-rel and x-relΔ29 messages. This could also relate to cell adhesion, or transformation-related processes since rel is a proto-oncogene. The least extremely disrupted embryos, which were

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injected with low concentrations of x-relpSP64T and x-relΔ29pSP64T message, appeared externally to have more or less normal heads, formation of a tail to some extent and a poorly extended mid-axis, which had an upward curve. These observations were verified by histological sections; internally the head looked well organised, the mid-axis had normal tissues, but they were unsegmented and their organisation was disrupted, and there was a small nervous system.

Overexpression of a x-rel putative dominant negative mutant produces almost the opposite phenotype, where the head and tail structures are most disrupted. If x-relΔ221 is a dominant negative, and only interferes with endogenous x-rel activity (and not that of other rel proteins) this might indicate that the highest concentration of x-rel is expressed in the head and tail. Following low concentrations of x-relΔ221 message, the head was a little disrupted, but the tail was usually far more seriously affected. The middle region of these embryos seemed much more normal. Histological sections showed that the internal organisation of the head was disrupted. In addition, in the posterior region of the embryo, the somites were particularly disorganised and tissues were tending to blend together rather than differentiating to form separate regions. In contrast, the internal organisation of the middle of the embryo was fairly normal. From these data,

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one might postulate a role for x-rel in the specification of the anterioposterior axis.

13.4.2 How specific is the aberration to development caused by overexpression of x-rel messages ?

It is possible that the phenotypes observed as a result of x-rel overexpression result directly from a disruption of the activities of other kB-binding proteins. Excess, ectopically-expressed x-rel protein may compete for binding sites or form non-functional heterodimers; x-rel may form heterodimers that have an altered function, whereas x-rel Δ 221 may form heterodimers that completely inhibit the function of other kB-binding proteins. However, this seems unlikely to be the whole explanation of the phenotypes since if this were the case x-rel, x-rel Δ 29 and x-rel Δ 221 might be expected to give similar phenotypes, which is not the case. It can be argued that since the phenotypes of overexpression and of a putative dominant negative mutant are opposite, that the phenotypes correlate to the natural functions of x-rel. However, two binding activities, Rel A and Rel B (see Chapter 11), which are distinct from an x-rel homodimer, are present in the embryo and bind to common sites. X-rel could, therefore, potentially disrupt these activities. However, the dimerisation of these activities with x-rel has not been demonstrated, and in any case, the DNA binding sites used in vitro (which were mammalian NF-kB and

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Drosophila dorsal binding sites) are not necessarily the natural DNA binding sites; it is possible that x-rel may not bind to the natural DNA binding sites of Rel A and Rel B. Another example of proteins that bind to common sites include the retinoic acid receptor and thyroid hormone receptor, but some sites are specific for the retinoic acid receptor and some are specific for the thyroid hormone receptor (Naar et al., 1991; Umenson et al., 1991).

From work on mammals, it is expected that x-rel naturally occurs as a heterodimer, perhaps with a rel-related protein and so homodimer overexpression may represent overexpression of an unnatural activity. X-rel is most similar to p65 (56.5% overall; 77.5% in the N-terminal region), which occurs as a heterodimer with another rel-related protein, p50. Perhaps a p50-like protein exists in Xenopus. No other proteins that bind to kB sites have yet been characterised in Xenopus. However, from GEMSA studies (see Chapter 11 and 12), additional binding activity, referred to as Rel A and Rel B, was detected. X-rel homodimers were not detected in band shift assays, but the possibility exists (and needs to be investigated further) that x-rel protein is a component of Rel A (see Chapter 12). However, the distribution of x-rel activity, suggested by the phenotype data does not correlate with the distribution of Rel A binding activity (which is most highly expressed in the anterior and middle regions) described in Chapter 12.

The block in gastrulation, observed as a result of

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injecting the highest concentration of x-relSP64T message, could be as a result of the enormous excess of this protein being produced. It is possible to block gastrulation by blocking RNA synthesis with RNA polymerase II inhibitors so that new zygotic transcription cannot occur; although the dorsal lip does not form in this case (Newport and Kirschner, 1982a). Overexpression of transcription factors in mammalian cells can lead to an effect, referred to as 'squenching' (Ptashne, 1988). This term describes a non-specific block of transcription as a result of interaction of an overexpressed transcription factor with components of the transcriptional initiation complex not in contact with DNA (Mitchell and Tjian, 1988). It is possible that a similar phenomenon may have occurred in the embryos overexpressing x-rel. The fact that injection of x-rel Δ 221pSP64T message does not cause a block in gastrulation would correlate with a squenching effect caused by overexpression of x-rel since x-rel Δ 221 does not contain the activation domain, which would be the main region that interacts with the components of the transcriptional initiation complex. However, to determine if this was a problem in the x-rel message injection experiments, the amount of new transcription and DNA replication occurring in the phenotype embryos was analysed. Table 9 shows that incorporation of ^3H -thymidine into DNA and ^3H -uridine incorporation into total RNA and poly (A)⁺ RNA of embryos that had been injected with x-relSP64T message, was not

Table 9.

Embryos injected with x-relpSP64T at the 2-cell stage and control, uninjected embryos were injected with ^3H -thymidine or ^3H -uridine (both at 10 mCi/ml) at stage 10 $\frac{1}{2}$ and incubated for 3 hours. Following this, 4 embryos (control and injected) from the ^3H -thymidine labelling and 6 embryos (control and injected) from ^3H -uridine labelling, were homogenised in 300ul of Kressmans' buffer. Homogenates were extracted twice with an equal volume of a 1:1 mixture of phenol/chloroform.

% incorporation was determined as follows:

a) total counts (^3H -thymidine and ^3H -uridine).

Duplicate 5ul samples of extract were pipetted onto a Whatman GF/C filter and the dried filter placed in scintillant and counted.

b) Total incorporation (^3H -thymidine and ^3H -uridine).

Duplicate 10ul samples of extract, 50ul salmon sperm DNA (1mg/ml), 100ul 100% (w/v) TCA and 1ml of water were mixed and incubated on ice for 10 minutes. The mixture was filtered through a GF/C filter, the filter was washed with 10ml of 5% (w/v) TCA, and then the filter dried, placed in scintillant and counted.

c) Incorporation into poly(A)⁺ (^3H -uridine only).

Nucleic acids were precipitated from 250ul of extract by the addition of 25ul of 3M NaAc (pH 6.5) and 500ul of ethanol. Pelleted nucleic acids were counted, and then suspended in 450ul of water. To this, 450ul of primer extension hybridisation buffer (see Methods section 3.10), and 1mg of oligo dT cellulose was added. The mixture was shaken vigorously for 4 hours, and the resin pelleted by microfugation. The supernatant (poly(A)⁻ fraction) was counted. The pellet was washed twice with 500ul of 2x SSC, and the poly(A)⁺ RNA eluted in 500ul of water at 100°C for 10 minutes. Resin was pelleted and the supernatant (poly A⁺ fraction) was counted.

Replication and transcription in injected embryos			
	[³ H] thymidine incorporation	[³ H] uridine incorporation into total	proportion of RNA incorporation into poly(A)+
Uninjected embryos	13.6%	2.6%	4.1%
X-rel64T injected embryos 500ug/ml	9.5%	1.8%	7.8%

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markedly different from that of uninjected embryos. New DNA replication and zygotic transcription, in general, is therefore not affected to a significant extent.

Furthermore, fractionation of radioactive RNA shows that the level of poly A⁺ RNA is not decreased and thus new transcription of mRNAs by RNA polymerase II is not generally squelched.

The block in gastrulation may reflect a more specific block by x-rel in the transcription of genes that are perhaps involved in the gastrulation process. Since x-rel Δ 221 does not inhibit gastrulation or produce a similar phenotype to the full-length message, over-expression of full-length x-rel is probably not solely inhibiting another rel protein. X-rel may specifically prevent a transcription factor or factors from binding to their target genes, which may be involved in the determination of anterioposterior position of mesoderm. X-rel is expressed maternally and its expression is increased until it reaches its peak at early gastrula (see section 6.2), so it is conceivable that x-rel has a role in gastrulation. If over-expression of x-rel did cause serious non-specific problems in gastrulation, one might expect that anterior and posterior positional values would be altered, although the outcome expected depends on ones interpretation of existing data on the way that the anterioposterior axis forms at this time. However, many of the moderately abnormal embryos that were injected with x-rel messages, have eyes and a

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sucker, suggesting the anterior mesoderm has not been ablated or posteriorised.

13.4.3 Further experiments to ascertain the role of x-rel in development.

To fully characterise the tissues that were scored by examination of whole embryos and sections, there is a pressing need for using molecular markers. This could be carried out using antibodies specific for certain tissues of the embryo (eg. gut specific or muscle-specific or neural-specific antibodies). However, since it is clear that it is primarily organisation, rather than presence or absence of tissues that is primarily affected, a different approach is likely to be productive. This involves probing RNA from the embryos with molecular markers of positional organisation, either using whole embryos, dissections or in situ. It would not only be interesting to see if these markers are expressed from a point of view of what tissues are present, but also because expression of x-rel may be directly linked to expression of some of these genes. If, as postulated, x-rel is involved in the specification of the AP axis, one might predict that its expression is related to the activation of homeobox genes. Many homeobox genes that display an anterioposterior gradient of expression are expressed in the neuroectoderm (see table 3). XlHbox1 is expressed in the anterior neural tube, neural crest and mesoderm; xlHbox6 is expressed in the

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posterior neural tube and lateral plate mesoderm. The murine homologue of zen, a zygotic gene repressed by dorsal in Drosophila, is found in the branchial and more posterior region of the head (Hunt et al., 1991). However, xhox3 is expressed early on in an anterior-posterior gradient of axial mesoderm. Since the character of the mesoderm determines the character of the neuroectoderm, x-rel could be having an effect on the activation of later neuroectodermally-expressed homeobox genes.

If x-rel were like dorsal, which is now not entirely expected from the results presented earlier, one might predict that the levels of xsna and xtwi would be elevated. However, I believe that it is possible that there is a Xenopus homologue of dorsal that is more homologous than x-rel, which would have a role in the activation of xtwi and xsna, if indeed the situation is analogous to that in Drosophila, where dorsal directly activates twist and snail.

If x-rel does have a function in AP axis formation then one might predict a graded distribution of x-rel expression, possibly at the nuclear translocation level. As argued earlier, the phenotype data may suggest that the highest expression of x-rel is in the posterior, with some in the head and not very much if any in the middle region. UV-treated embryos, which are posteriorised, showed a fivefold increase in the level of xhox3 expression (Ruiz i Altaba and Melton, 1989b). Analysing the effect of UV and

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lithium on x-rel expression may be complex; perhaps it would be increased in UV-treated embryos, but slightly decreased in lithium-treated embryos. It is possible that x-rel is expressed in the extreme anterior and posterior ends of the embryo. It would be interesting to examine the effect of x-rel on xhox3, as well as Xlhbox1 and Xlhbox6.

In view of a putative role for x-rel in the specification of the AP axis, it would be interesting to determine whether the effects of retinoic acid and growth factors would be modified by x-rel over-expression or expression of x-rel $\Delta 221$, when they are applied to transplanted animal caps. Retinoic acid has been shown to cause an anterioposterior transformation in the developing CNS of early X. laevis embryos (Durstun et al., 1989; Sive et al., 1990). Growth factors have been shown to cause an increase in the expression of Xlhbox6 in transplanted animal caps; this effect was augmented by retinoic acid (Cho and De Robertis, 1990). A posteriorisation of the CNS was therefore occurring since Xlhbox6 is expressed specifically in the posterior region of the CNS. This effect may be, in part, caused by RA affecting other processes such as the anterioposterior specification of the mesoderm. The effect on x-rel expression after treatment of embryos with RA, which may be stimulated by the addition of growth factors, may indicate whether or not it is a component of the biochemical pathway of generating AP axis formation.

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Chapter 14.

Results and General Discussion.

I have isolated a cDNA for a Xenopus laevis rel transcription family member from a matured oocyte cDNA library, which I have isolated and called x-rel. This cDNA has been completely sequenced and shown to have a full-length coding region. The original aim was to isolate a cDNA for a gene that had a function in Xenopus analogous to that of the Drosophila rel family member, dorsal. Rel family members all share a highly conserved N-terminal region, which is responsible for DNA binding and dimerisation. The C-terminal domains of different rel family members are not conserved. X-rel has the characteristic rel homologous domain at the N-terminal end. The C-terminal end of x-rel shows no homology to dorsal. However, unlike other rel family members, x-rel also shares a significant level of homology with the NF-kB constituent, human p65, in its C-terminal end, and is probably the Xenopus homologue of p65. NF-kB which consists of two rel protein subunits, p50 and p65, has a wide range of functions, the best characterised of which is the activation of immunoglobulin genes during lymphoid cell maturation.

Messenger RNA synthesised from x-rel constructs translates in vitro to produce an approximately 60kD product, a size consistent with sequence data. In vivo translation of micro-injected synthetic messenger RNA in

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oocytes and embryos also produces a protein of this size which is translocated to the nucleus in both oocytes and embryos. Translocation to the nucleus is not significantly affected by C-terminal deletions of x-rel protein. This is in contrast to the increased nuclear localisation of C-terminally deleted dorsal and chicken c-rel proteins.

Gel electrophoretic mobility shift assays (GEMSA), using extracts from oocytes and embryos which had been micro-injected with synthetic wild-type and mutant x-rel messages, indicated that x-rel protein binds to DNA at sites related to those bound by NF-kB in the immunoglobulin k chain enhancer (kB sites). Dorsal and human p65 homodimers also bind to these sites. Furthermore, x-rel, like p65 and other rel proteins, binds as a dimer, since an intermediate mobility binding activity was detected when using extract from oocytes that had been co-injected with wild-type x-rel and deleted x-rel synthetic transcripts, compared to the binding activities observed using extract from oocytes which had been independently injected with these transcripts.

A Northern blot, using poly A⁺-selected RNA, indicated that x-rel is a maternal message whose peak level occurs just prior to the onset of gastrulation (at about stage 9), then decreases to lower but detectable levels in later embryogenesis. An even dorsal to ventral distribution of messenger RNA was shown for neurula embryos; work is in progress to augment the data on the spatial distribution of

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messenger RNA in early embryos. Assuming the level of messenger RNA reflects the level of protein, this distribution is not indicative of a role in Xenopus analogous to that of dorsal in Drosophila, which is involved in the specification of the dorsal-ventral axis. The peak level of x-rel correlates better to the determination of the anteroposterior axis. This correlates with sequence data suggesting that x-rel is not a dorsal homologue, but a p65 homologue. X-rel has a peak level before that which would be expected for a p65 homologue, which is primarily involved in lymphoid tissue differentiation. X-rel may have a role similar to p65 later in development, and also an earlier developmental role. It is not known whether or not x-rel functions at this stage as a homodimer, or in association with, for example, a p50 homologue. No p50 homologue was detected either in the low stringency screen or Northern. Endogenous x-rel has not been detected in GEMSA (see below), and so it is not known if x-rel occurs as a homo- or heterodimer. In mammals p65 has only been reported in association with p50 (although p50 homodimers, the role of which is not known, do occur). X-rel homodimers (and p65 homodimers) have binding activity and may have a function in this form.

I was unable to detect endogenous x-rel protein in X. laevis extracts on Westerns with a polyclonal antiserum raised against a region of x-rel protein. In addition, endogenous x-rel homodimers were not detected and mixed

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dimers containing x-rel were probably not detected using GEMSA. This may reflect a low abundance of x-rel proteins. Consequently, it was not possible to compare the nuclear-cytoplasmic distribution of x-rel protein and to determine if there was a mechanism of anchoring in the cytoplasm analogous to that of other rel family proteins (see Introduction section 1.3.4). An antibody with greater affinity for endogenous protein should clearly be a future goal in order to help answer these questions. Overexpressed x-rel protein enters the nucleus, but may saturate a postulated cytoplasmic retention mechanism.

Two endogenous binding activities were detected, using GEMSA, in extract from Xenopus oocytes and early embryos. Although able to bind to kB-like enhancer sequences, these activities appeared not to represent x-rel protein, nor x-rel protein in association with another factor as a heterodimer. The endogenous binding activities represented by Rel A and Rel B have not been characterised and no work has been undertaken to ascertain their functions in development, although this would be a possible branch of future work. It is not known whether these activities represent rel family members. Rel A peak activity is detected at stage 9 where it is enriched in the animal region and levels off at later stages where it shows an even distribution. Rel B activity is first detected at stage 9 and maintains a high level of activity through later embryogenesis.

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The experiments carried out to determine the function of x-rel in early X. laevis development were based on the ectopic expression of both wild-type and deleted x-rel proteins. X-rel transcripts were synthesised from a high expression vector, pSP64T, and injected into embryos at the 2-cell stage. ³⁵S-methionine labelled-embryos were used to ensure that expression of these transcripts continued at a high level through gastrulation. Evidently, to interpret the phenotype resulting from expression of these transcripts, control synthetic transcripts it was necessary to inject in each experiment. Uncapped and antisense message were injected for controls and this usually resulted in normal embryos. Injection of a putative dominant negative, x-relΔ22lpSP64T, also served as an internal control for injection of the wild-type transcript (x-relpSP64T). This was demonstrated by the complementary phenotypes observed as a result of injection of both of these transcripts. Occasionally, a similar amount of synthetic retinoic acid receptor message was injected; these embryos developed completely normally (data not shown). These controls indicated that the phenotype resulting from x-rel-injected embryos was due to a specific activity of the protein encoded.

The phenotypes observed from x-rel-injected embryos were characterised according to their external appearance and histological analysis of later-stage embryos. Injection of high levels of full-length x-rel and x-relΔ29 (lacking 29

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C-terminal amino acid residues) transcripts resulted in a block in gastrulation. At lower concentrations, those proceeding through gastrulation displayed a consistent disorganisation of their nervous systems, somites and a lack of migration of melanocytes; also they often had epidermal thickenings. Disorganisation of these structures was most apparent in the middle of the embryos and the mid-axis was often reduced, whilst head and tail developed relatively normally. Certain regions of the embryos, such as the notochord and endoderm, were consistently normal and the dorsal-ventral organisation of embryos was preserved.

Synthetic transcripts of a putative dominant negative were also injected. This clone, x-relΔ221, which lacked 221 amino acids of the C-terminal activation domain, but retained the N-terminal DNA binding and dimerisation domains. In contrast to the full-length x-rel and x-relΔ29 injections, injection of x-relΔ221 transcripts never resulted in a block of gastrulation, in fact, over-gastrulation resulting in a cavity at the blastopore, was observed. At later stages, those tissues disorganised following x-rel and x-relΔ29 injection, were also disorganised following injection of x-relΔ221 transcripts. However, the spatial arrangement of this disorganisation along the anteroposterior axis was distinct. The mid-axis was least affected, with the head and tail showing most tissue disorganisation, and in extreme cases truncation of the head and tail was observed. As with x-rel and x-relΔ29

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injections, x-rel Δ 221 injections did not affect the notochord, endoderm or the dorsal-ventral organisation of the embryo.

These results may suggest a role for x-rel in establishing anteroposterior positional values. Perhaps, since the wild-type has a greater effect on the mid-axis and the putative dominant negative has a greater effect on the anterior and posterior ends of the embryo, x-rel has a role in the specification of both anterior and posterior positional values. Recent data from our laboratory (Hugh Woodland, pers. comm.) suggests that x-rel does, indeed, act as a transcriptional activator, and x-rel Δ 221 as a dominant negative mutant. Co-injection of transcripts with a CAT reporter gene containing a kB site in the promoter, indicates that x-rel activates the reporter gene, whereas x-rel Δ 221 represses the reporter (presumably by blocking endogenous kB binding factors). The activation of the reporter gene by x-rel can be blocked by co-injection of x-rel Δ 221 transcripts. Nevertheless, it is possible that some of the effects of x-rel injection (both full-length and truncated proteins) result from the disruption of other kB binding proteins (Rel A and Rel B, at least, are present, and bind to sites in common with x-rel), and that the x-rel homodimer produced by injection of synthetic message is not a naturally occurring binding activity (mammalian p65 is not known to occur naturally as a homodimer).

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It is vital to analyse x-rel-micro-injected embryos at the molecular level using tissue- and position-specific markers (for example, homeobox genes). In addition to establishing a more accurate picture of the effects of message injection, this may identify some of the target genes of x-rel.

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Cell 61: 255-265.

Appendix.

Sequences of primers referred to in text.

Arrows are written above the sequence contained in the primers, and in the 5' to 3' direction. Primer 1 has the extension 5' GACTAGATCT 3' at the 5' end and primer 2 the extension 5' GACTGGATCC 3' at the 5' end.

0001 TATCTATTAGGGAAAGGAAGAAGAGGGGAGCGAAACCGTGAAGTGGAGAAGTAAAGAGTATCGGTGGCGAAAATATGTATACAT
 Ape ← X-rel.2 Jpe → Primer 1
 0091 TCTGAAACTGCAAGGGGAGCTAGCCCCCTGTTAAAGGCCAGCGTTGAACATGGATGGATTCCATTGGACAGACATAGTTTCAAGCATGCCT
 M D G P H W T D I V S S N P

 0181 CCTOCATCCCACTGTGGAAATTATAGAGCAGCCAAAGCAGCGGGATGCGGATTCGTTACAAAGTGTGAAGGGCGCTCTGCTGGAAAGC
 P S I P P V E I I E Q P K Q R G N R F R Y K C E G R S A G S

 0271 ATTCAGGTGAACGAAGCAGGACACATCAAAAATCAACCCACTATTAAGATCAACAATTAACGAGGCCAGCTCGTATTCGGATATCT
 I P G E R S T D T S K T H P T I K I N N Y Q G P A R I R I S JPCR

 0361 CTAGTTACAAAGGATTCCTCACATAAACTCAACCTCATGAGTTGGTTGGAAAGGACTGCAAGGATGGATATTATGAAGCTGAACTTCC
 L V T K D S P H K P H P H E L V G K D C K D G Y Y E A E L S

 0451 CCAGATCGGAGTATCCACAGCTTCCAGAACTGGGCATCCAGTGTGTGAAAAAGAGAGAGGTGGAAGATGCCCTCGCCCATCCGATTCGG
 P D R S I H S F Q N L G I Q C V K K R E V E D A V A H R I R

 0541 ACAATAATAACCCCTTTAATGTGTCTCTGAGGAGCTGAAGGCGGATTACGACCTCAACAGGTTTGGCTGTCTCCAGGTTTTCATT
 T N N N P P N V S P E E L K A D Y D L N T V C L C F Q V F I

 0631 CCTGATCAGGCTGCTGGTCCGATGCTTCCATTACCAATTTGGTATCCAGCCCATCTATGACAAACAGAGCCCCATAACTGCCGAACTG
 P D Q A A G R N L P L P F V V S Q P I Y D N R A P N T A E L Jhinc2

 0721 AAGATATCGCAGTGAACAAAAATCCGGGAGCTGCTGGGAGGAGTGAATTTCTTACTTTGTGATAAAGTACAAAAAGATATCGAG
 K I C R V M K N S G S C L G G D E I F L L C D K V Q K D I E

 0811 GTGATATTCGGTTTGGGCACTGGGAAGCTCGTGGGATATTTTCCAAAGCAGATGTTACCGCCAGGTGGCTATTGCTTCCGAAACCCCT
 V I F G L G N W E A R G I F S Q A D V H R Q V A I V F R T P

 0901 GCTTTTCAAGACACAAAAATACGTCACTGTGCAAAAGTCAAAATGACAGCTGAGGCGCCATCAGACAAAAGTCACTGAGCCTATGGAG
 A F Q D T K I R Q S V K V Q M Q L R R P S D K E V S E P N E

 0991 TTTCAATATCTTCCAGATGAAGGAGACCCCAACATATTTGATGAAAAACGAAAAAGGACCCCTTGACAAATTTCAAGCATTATGTGAAAAAT
 F Q Y L P D E G D P H H I D E K R K R T L D N F K H Y V K N

 1081 AATCCTTTTGCAGGAGGAAACGGGACCCCAACGAGAAATGCTGTGGCAAAATGTAATGTGCCAAACAAATGAGAGCCTATAAGAGCC
 N P F A G G E T R P Q R R I A V A N R N V P T K S E P I T P

 1171 AGCATACCCGTTCCAAATCCAGTGGTATCCTGTCTTCCATTTTCCATGCCCGTTTTAAAGGCTGAGAAATGTTACTTCACTTCCAGCTCTT
 S I P V P N P V V S C L P P S N P V L K A E N V T S P S T L

 1261 CTGTCCACCGTCAACATCAGGACTTCCAGCAATTTAGGATTTTCAACAGCCCTCCCTCTCAGTCTGATCAGACCCGCTTAGAATCCATG
 L S T V N I S D F S N L G F S S Q P P S Q S D H D R L E S N

 1351 TTAAACTACCCCTCATTCAGGCGATGCAAAATCTGGATTTAGTAGAAATGTTGCCACATGAGAAACGAAAGCCGATGTACCAAGTTGAGT
 L N Y P S P P G D A N L D L V E N L P H E M E S R C T S L S

 1441 TCTATTGACAATAGTATTTCAGCCAACTTCTCAGCGAGTCAAAATCACTGGGCACTCTCTGCACTTTTACAAGAGCCTGGGACTAGT
 S I D N S D F S Q L L S E S Q S S G T L S A A L Q E P G T S

 1531 CAAGCCAGTTCATGGCTTATCTCTGAATCCATTTGCTCGACTTATGACCAACCGGCCAATGAGGATGAGGGTGGAGAGAAATGACAGT
 Q G T F M A Y P E S I A R L N T N R P N E D E G G E R I D S

 1621 GGCCTGATTAATGGATGTTGATATGACGAGGAGGAGATTCAAACTCCTTATTGAGCTTGACTTTCTTCTTATTAAGTAAAC
 G L I N G N F D N Q Q G G D S L T S L F E L D F S S L L S N primer 2 ←

 1711 ATGAAATAAATGCTTTATTAATATGAGCAATAAGACTAAGCATGAGAGCTGCCTAAATGCTGTGGTTCCGGATGTTGTTCAAATGC
 N K X-rel.2 poly A

 1801 AGTAGCCAGAAATGTGCAATGATCACTTCTTCAACCTCTTAAGACTCTGTAGATACTTTATCAAGATATTTTAAAGTTATAITTC A

 1891 CCTCTGCTACTATCATGCAGAAATAAATAAGCATGGTCTTAAAAA

