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Developmental effects of over-expression of normal and mutated forms of a *Xenopus* NF-κB homologue

Jill C. Richardson, Derek Gatherer, Hugh R. Woodland*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

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Abstract

High level over-expression of *XrelA1*, a homologue of the p65 sub-unit of NF-κB and of *Drosophila* dorsal, arrests *Xenopus* development at the gastrula stage, producing a reduction in the levels of expression of various genes of developmental interest without general reduction in transcription or cessation of cell division. There is little *Goosecoid* expression, even though a dorsal lip forms. At lower levels *XrelA1* mRNA primarily produces disruption of the mid-dorsal axis. A dominant interference gene product, Δ222, produces mainly posterior, but also anterior abnormalities. On the basis of these results we postulate that the role of *XrelA1* in the vertebrate embryo is unlikely to be in dorsoventral development, but more likely in the formation of the termini.

Keywords: Xenopus; NF-κB; Dorsal; Axis formation

1. Introduction

We and others have recently isolated a number of closely related cDNAs (*XrelA1* and *A2*), probably polymorphic variants of a single gene, that are expressed in oocytes and all subsequent embryonic stages of *Xenopus* (Kao and Hopwood, 1989; Richardson et al., 1994). They are most closely related to the RelA (p65) component of mammalian and avian NF-κB. This is a transcriptional activator acting on a number of genes expressed in lymphocytes, such as certain immunoglobulins and the HIV LTR, and its biology has been frequently reviewed (e.g. Baeuerle, 1991; Nolan and Baltimore, 1992; Liou and Baltimore, 1993; Beg and Baldwin, 1993). NF-κB is composed of two subunits, in mammals originally called p50 and p65, but now known as NF-κB1 and RelA, respectively. Both are involved in DNA binding, but RelA has a greater effect on transcriptional activation and NF-κB1 has tighter DNA binding. In B lymphocytes NF-κB is constitutively active, but in the majority of cells, including T lymphocytes, it is usually inactivated by association with IκB (itself a family of molecules), which primarily acts by inhibiting nuclear translocation of NF-κB. Activation of the IκB/NF-κB complex can occur by any one of a very wide range of stimuli and involves dissociation of IκB from the complex, and unmasking of the NF-κB nuclear translocation signal. The NF-κB subunits are part of a wider family of transcription factors, including also Rel, the product of the *c-rel* gene, in vertebrates and dorsal in *Drosophila*.

The existence of dorsal in *Drosophila* was the original stimulus for initiating our study. The development of ventral structures in this fly depends on the generation of a gradient of active dorsal protein translocated into the ventral nuclei (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). This activation is achieved by the dissociation of the product of the *cactus* gene from dorsal; cactus is homologous to IκB (Geisler et al., 1992; Kidd, 1992; Govind and Steward, 1993). In the *Drosophila* blastoderm the targets for *dorsal* include the genes *twist* and *snail*, both of which have homologues present at high levels around gastrulation in *Xenopus*, *Xtwi* and *Xsna* (Hopwood et al., 1989; Sargent and Bennett, 1990). In certain ways the dorso-ventral development of *Drosophila* equates to that of *Xenopus* in an inverse fashion, in that the dorsal tissues of *Drosophila* and the ventral tis-

* Tel.: +44 203 523536; Fax.: +44 203 523701; E-mail: hw@dna.bio.warwick.ac.uk.

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sues of *Xenopus* develop by default when maternal morphogens are not activated. In *Drosophila* the activation of ventral development occurs via the Toll transmembrane receptor, and the extra-cellular signal is transmitted within the ovarian follicle (St Johnson and Nüsslein-Volhard, 1992). In *Xenopus* the initiation of dorsal development involves a cytoplasmic contraction polarised by the entry of the sperm, but the molecules immediately affected by this movement are unknown. There are other homologous molecules active in dorso-ventral development in both species; for example, neural/epidermal differentiation in *Drosophila* depends on the secretion of the product of the *Dpp* gene, whose product is a member of the TGF-β family (Ferguson and Anderson, 1992) and both dorsal and ventral mesoderm induction in *Xenopus* involves members of this family (Hemmati-Brivanlou and Melton, 1992). Nevertheless, without further information we cannot say that parallels between the systems represent homologies, rather than analogies. Since dorsal plays a pivotal role in dorso-ventral axis formation in *Drosophila* we have tried to investigate whether homologous molecules are involved in forming this axis in *Xenopus* development.

We have previously shown that in developing embryos, as postulated from the behaviour of mammalian RelA/p65 in cultured cells, XrelA1 wild-type protein at low and intermediate levels of over-expression will stimulate HIV LTR-driven transcription in a process dependent on the presence of a κB site. However, at high concentrations, transcription of at least one reporter gene was inhibited (Richardson et al., 1994). Broadly similar results were obtained with XrelA1 protein lacking the C-terminal 29 amino acids, although the activation efficiency was a little reduced. In contrast, a construct in which the entire activation domain was deleted did not increase κB-dependent transcription, but rather, at high concentrations it inhibited the action of the wild-type protein. This protein is therefore a dominant negative inhibitor of XrelA1 activity. In this paper we report the effects of over-expressing XrelA1 and the dominant negative mutant, both on the way that embryos develop and the extent to which a number of developmentally interesting endogenous genes are transcribed.

2. Materials and methods

2.1. Biological materials

Embryos were obtained, cultured and micro-injected as described by Richardson et al. (1994). The in vitro transcription of plasmids to give synthetic mRNA for injection was also as described previously.

2.2. Sources of probes for RNase protection and in situ hybridisation

The clone containing *Xenopus brachyury* was provided by Dr Jim Smith (*pXBra*, Smith et al., 1991). Probe for RNase protection was produced by cutting with *Ssp*I and for in situ hybridisation by cutting with *Eco*RV and transcribing with T7 polymerase in both cases. The clone containing *Evx-1* (*Xhox-3*) was provided by Dr Jeremy Green (*pXHox-3ΔB*; Ruiz i Altaba and Melton, 1989). A probe for RNase protection was produced by cutting with *Alu*I and transcribing with T7 polymerase. The clone containing *Goosecoid* was provided by Dr Eddy de Robertis (*pΔgsc;* Cho et al., 1991). Probe for RNase protection was produced by cutting with *Sma*I and transcribing with T3 polymerase. The clone containing *Hox-B9* (formerly *XlHbox6*) was provided by Dr Colin Sharpe (*pG1s#104*; Wright et al., 1990). Probe for RNase protection was produced by cutting with *Nci*I or *Sma*I and transcribing with T7 polymerase. The clone containing *Krox-20* was provided by Dr David Wilkinson (*5′XKr20*; Bradley et al., 1992). Probe for in situ hybridisation was produced by cutting with *Eco*RI and transcribing with T7 polymerase. The clone containing *Noggin* was provided by Dr Richard Harland (*pnogginΔ5′*; Smith and Harland, 1992). A subclone for RNase protection was generated by excising the smaller *Bam*HI-*Eco*RV fragment, polishing the ends and religating (*nogginΔ5′-230*). A probe for RNase protection was produced by cutting this subclone with *Eco*RI and transcribing with T7 polymerase. The clone containing an ornithine decarboxylase gene fragment was provided by Dr David Tannahill (*ODC(*)*; Isaacs et al., 1992). Antisense probe for RNase protection was produced by cutting with *Bgl*II and transcribing with T3 polymerase. The clone containing *pintallavis* was provided by Dr Ariel Ruiz i Altaba (*pF5*; Ruiz i Altaba and Jessell, 1992). A subclone for RNase protection was generated by excising the smaller *Pst*I fragment, and religating the remainder (*pF5ΔPst*) A probe for RNase protection was produced from this subclone by cutting with *Xmn*I and transcribing with T7 polymerase. The clone containing *snail* was provided by Dr Michael Sargent (*xsna-pSP7*; Sargent and Bennett, 1990) and the probe for RNase protection was produced by cutting with MspI and transcribing with SP6 polymerase. The clone containing *twist* was provided by Dr John Gurdon (*t18-pSP73*; Hopwood et al., 1989). A probe for in situ hybridisation was produced by cutting with *Hpa*I and transcribing with T7 polymerase. The clone containing *Xenopus Wnt-8* was provided by Dr Richard Harland (*pGEM5R-Xwnt-8*; Smith and Harland 1991). 3′ sequences were removed by digestion with *Hind*III and *Not*I and the largest fragment was religated to generate *pXwnt-8Δ3′*. Probe for RNase protection was produced by cutting with *Eco*RV and transcribing with T7 polymerase.

Whole mount in situ hybridisation was by the method of Harland (1991).

2.3. RNA extraction and analysis

Embryonic RNA was prepared by homogenising embryos in groups of 10, using an Eppendorf tube and a

plastic pestle, in 0.5 ml of XTB (300 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 1% SDS, 0.6 mg/ml Proteinase K, Boehringer Mannheim). An equal volume of phenol was added followed by vigorous vortexing. The tube was left on ice for 10 min with brief vortexing every minute, then spun in a microcentrifuge. The upper layer was re-extracted with phenol, then chloroform/isoamyl alcohol (24:1) and precipitated with 2 vols. of ethanol at −20°C for 1 h, resuspended in 0.5 ml XTB and incubated at 37°C for 15 min, followed by re-extraction and reprecipitation as above. The dried pellet was resuspended in 20 μl water and stored at −70°C.

Radiolabelled RNA probe was prepared by transcribing linearised antisense template DNA with T7, SP6 or T3 polymerase (Gibco BRL) according to the manufacturer's instructions, incorporating 50 μCi of [α-^{32}P]UTP per reaction. Full-length probe was separated from unincorporated nucleotide by electrophoresis on an 8% polyacrylamide gel under standard conditions. The region of the gel containing the probe was excised, homogenised in XTB and eluted in XTB at 37°C for 90 min, then phenol extracted and precipitated using yeast tRNA as a carrier. The pellet was resuspended in 50 μl of water and stored at −20°C.

Hybridisations were carried out in 50 μl of 40 mM Pipes (pH 6.4), 1 mM EDTA, 400 mM NaCl, 80% deionised formamide, to which were added 5 μl of embryonic RNA (approx. 2 embryo equivalents), and 2 μl probe (excess), in an oven at 46°C for 4 h. RNase digestions were performed by adding 500 μl of RNase digestion mixture (300 mM NaCl, pH 7.4, 10 mM Tris, 5 mM EDTA, 3 μg/ml RNase A (Sigma), 100 U/ml RNase T1 (Gibco BRL)), and incubating at 37°C for 1 h. Excess RNase was destroyed by addition of 50 μl 10% SDS, and 5 μl proteinase K (20 mg/ml, Boehringer Mannheim) and incubating at 37°C for 15 min, then extracting with phenol/chloroform/isoamyl alcohol and precipitating using yeast tRNA as carrier. The dried pellets were resuspended and electrophoresed on an 8% polyacrylamide gel.

Gels were fixed in 10% acetic acid, 10% methanol in water for 30 min, dried and exposed to film for 1 day or 1 week. If bands were observed, they were quantified on a Molecular Dynamics phosphorimager with ImageQuant software.

2.4. Histology and antibody staining

Sectioning of embryos for immunohistochemistry and staining with antibodies was performed as described by Jones and Woodland (1989). Whole mount antibody staining was by the method of Dent et al. (1989).

Conventional histology was done by fixing embryos in Bouin's solution, embedding in Paraplast and sectioning at 10 μm. The sections were stained with haemalum and light green.

3. Results

3.1. Effects of expression of very high levels of XrelA1

The wild-type coding sequence was transcribed from the plasmid *XrelA1pSP64T* in the presence of cap analogue to give capped mRNA that was stable and translationally active in the embryo, as described previously (Richardson et al., 1994). This RNA was injected into both blastomeres at the two cell stage. The activity of this RNA on the transcription of injected reporter genes was previously reported by Richardson et al. (1994). While the dose response curve varied quantitatively between RNA preparations, the shape of the curve was similar and we found that the transcriptional and morphological effects were broadly parallel to each other. In Tables 1A and 2 a number of experiments using RNAs that were effective only at higher concentrations are combined. Because of embryo and RNA variability, pooling makes the results less clear cut than presenting the results of an individual experiment; in Table 1B we show a single experiment with higher activity RNA. This was the preparation whose effects on the transcription of injected DNAs encoding chloramphenicol acetyl transferase was reported by Richardson et al. (1994).

At concentrations above 5 ng/embryo there was highly reproducible arrest of gastrulation. In most experiments the effect was evident in 100% of embryos, and when a very few embryos did develop beyond this stage, they were sufficiently normal to suggest that the injection technique was at fault. In affected embryos, development up to gastrulation was indistinguishable from uninjected controls, and the dorsal lip crescent of dark bottle cells invariably appeared in a normal fashion, but it soon stopped spreading ventrally, usually extending no more than 20% of the distance to the ventral marginal zone (Fig. 1A,B). Morphologically, development was otherwise normal up to stage 10, as judged by external criteria, then it was completely arrested until the controls reached the late neurula stage, when the blocked embryos died. The concentration of XrelA1 mRNA blocking gastrulation corresponds to that which inhibited a non-κB-dependent thymidine kinase promoter (Richardson et al., 1994), whereas continued abnormal development is seen at concentrations where κB-dependent transcription was strongly stimulated (see below). Where it was tested, the efficiency of preparations in stimulating reporter gene transcription correlated with their effects on development. Controls for this experiment included the same concentrations of antisense capped mRNA and uncapped sense transcripts, both transcribed from the same plasmid preparation as the capped sense mRNA. Neither mRNA produces detectable levels of protein product (Richardson et al., 1994) and neither affected gastrulation. In addition the effect was not seen with the mRNA transcribed from the *Δ222* clone (see below), which lacks the transcriptional activation domain. It is translated very well through

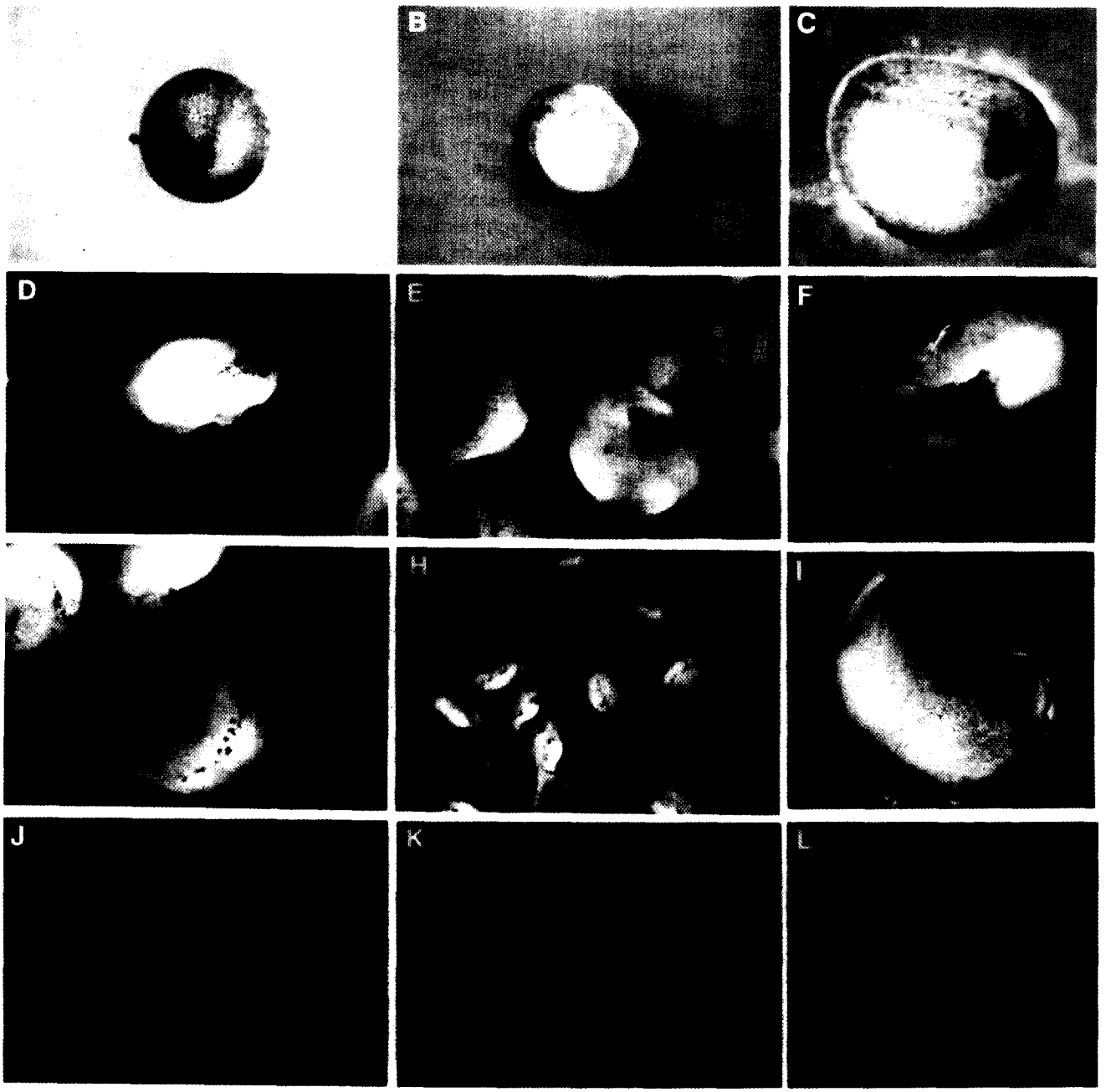

Fig. 1. Morphological appearance of embryos injected with mRNA encoding XrelA1 and its derivatives. (A) Posterior view of a normal stage 13 embryo injected with 10 ng uncapped XrelA1 mRNA. (B) Equivalent embryo injected with the same amount of capped RNA. (C) Equivalent embryo injected with 5 ng capped Δ222 mRNA. (D) Extremely affected stage 39–41 embryo injected with 2 ng capped mRNA; head and tail development are not apparent, but presence of a nervous system is signalled by the presence of a line of melanocytes. (E) Moderately to highly affected embryos at stage 30–33, injected with 2 ng XrelA1 capped mRNA. (F) Mildly affected stage 39 embryo injected with 1 ng capped XrelA1 mRNA; head and tail development are slightly abnormal, segmentation is affected in the mid axis and in addition there is a marked, dark, warty thickening of the epidermis (these thickenings are typical of mildly affected embryos). (G) Very mildly affected embryo injected with 2 ng capped XrelA1 mRNA, showing slight mid-axis abnormalities and growth of the main axial tissues through the end of the tail fin. (H) Stage 40 embryos injected with 10 ng uncapped Δ222 mRNA. (I) Stage 31 embryo injected with 10 ng capped Δ222 mRNA. (J–L) Sections of stage 30 embryos stained with the epidermal antibody marker 2F7. (J) Injected with 10 ng uncapped XrelA1 mRNA. (K,L) Injected with 2 ng capped XrelA1 mRNA showing in (K) fin-like extensions and epidermis within the embryo and in (L) the typical warty structures (see also F).

blastula stages and did not stimulate κB-dependent transcription nor, at high concentrations, inhibit the transcription of any clones tested that lacked κB binding sites (Richardson et al., 1994). The controls therefore support the view that the arrest of development was dependent on the presence of the transcriptional activation function in the highly over-expressed XrelA1 protein.

One possible effect of high levels of XrelA might have

Table 1A
Development of XrelA-injected embryos

RNA sample	No. of exp.	No. injected	Cleaving normally		Blocked at stage 10-10.5		Normal development		Later abnormal development		"Spina bifida"	
			No.	%	No.	%	No.	%	No.	%	No.	%
500 μg/ml XrelA	2	49	46	94	45	98	0	0	1	2	0	0
250 μg/ml XrelA	3	68	63	93	36	57	2	3	25	40	0	0
100 μg/ml XrelA	5	113	100	88	39	39	2	2	59	59	0	0
50 μg/ml XrelA	3	70	63	90	1	2	29	46	33	52	0	0
500 μg/ml uncapped XrelA	5	98	92	94	0	0	65	71	7	8	20	22
100 μg/ml uncapped XrelA	2	44	34	80	0	0	32	94	0	0	2	6
500 μg/ml antisense XrelA	2	41	39	95	0	0	33	85	1	3	5	13
500 μg/ml x-relΔ29	4	90	72	80	15	21	4	6	53	74	0	0
250 μg/ml x-relΔ29	3	63	51	81	12	24	5	10	32	63	2	4
100 μg/ml x-relΔ29	2	49	42	86	0	0	13	31	29	69	0	0
50 μg/ml x-relΔ29	1	25	16	72	4	22	9	50	5	28	0	0
500 μg/ml uncapped x-relΔ29	4	88	73	83	1	1	58	79	2	3	12	16
500 μg/ml x-relΔ222	2	41	34	83	0	0	0	0	25	74	9	26
250 μg/ml x-relΔ222	3	59	54	92	0	0	2	4	48	89	4	7
100 μg/ml x-relΔ222	2	40	36	90	0	0	17	47	19	53	0	0
50 μg/ml x-relΔ222	2	39	36	92	0	0	21	58	9	25	6	17
25 μg/ml x-relΔ222	1	20	17	85	0	0	12	71	5	29	0	0
500 μg/ml uncapped x-relΔ222	2	40	38	95	0	0	36	95	2	5	0	0

been to arrest DNA synthesis or to severely inhibit RNA synthesis. The latter is particularly relevant because it is known that the transcriptional inhibitors actinomycin D and α-amanitin block gastrulation, although the dorsal lip is not formed at all (Brachet and Denis, 1963; Wallace and Elsdale, 1963; Newport and Kirschner, 1982). To test whether there were any major effects of these kinds we first measured the incorporation of injected [^{3}H]thymidine. There was only a small reduction (Table 3), suggesting that the XrelA1 mRNA preparation did not have a major toxic effect. We also measured the incorporation of [^{3}H]uridine into total and poly(A)$^+$ RNA (Table 3). Any effect was apparently primarily on non-poly(A)$^+$ RNA (anucleolate mutant embryos make no rRNA but develop to a feeding stage; Brown and Gurdon, 1964). These measurements are fairly crude, but they do show that the blocked embryos are still alive and that DNA synthesis and RNA polymerase II function are not severely disrupted (see Section 4). More accurate measurements of general RNA synthesis were not made because the critical issues centre around the transcription of particular genes. This is considered below.

3.2. Morphological effects of injection of lower levels of XrelA1

Lower levels of capped XrelA1 transcripts do not arrest the gastrulation of embryos. In some experiments embryos show exogastrulation to variable degrees and as a consequence later develop with a split posterior axis made up of duplicated notochords, nervous systems and tails (for shorthand we refer to this syndrome as 'spina bifida', but we do not mean to imply an analogy with this

Table 1B
Development after injection with high activity XrelA mRNA

RNA sample	No. injected	No. cleaving normally	No. blocked at stage 10	No. reaching 'tailbud'		Of those reaching tailbud					
						Normal development		Later abnormal development		Spina bifida	
				No.	%	No.	%	No.	%	No.	%
2 ng/embryo XrelA	16	16	16	0	0	0	0	0	0	0	0
1 ng/embryo XrelA	16	16	16	0	0	0	0	0	0	0	0
500 pg/embryo XrelA	16	7	7	0	0	0	0	0	0	0	0
250 pg embryo XrelA	16	12	7	5	42	0	0	2	40	3	60
130 pg/embryo XrelA	16	16	3	13	81	0	0	13	100	10	77
60 pg/embryo XrelA	16	15	1	11	93	5	45	6	55	3	27
Uninjected controls	16	16	16	16	100	16	100	0	0	0	0

Table 2
Later survival of embryos injected with XrelA RNAs

RNA sample	No. of exp.	No. injected	No. reaching tailbud		Of those reaching tailbud									
					Normal		'Spina bifida'		Reduced head, curl in axis		No head, reduced axis		Little axis visible externally	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
500 μg/ml XrelA	2	49	1	2	0	0	0	0	1	100	0	0	0	0
250 μg/ml XrelA	5	108	13	12	4	31	1	8	1	8	1	8	6	46
100 μg/ml XrelA	6	123	73	59	15*	21	0	0	28	38	16	22	14	19
50 μg/ml XrelA	3	65	54	83	28*	21	0	0	21	39	2	4	3	6
500 μg/ml uncapped XrelA	4	79	67	85	44	66	19	28	3	4	1	1	0	0
100 μg/ml uncapped XrelA	2	45	29	64	16	55	10	34	3	10	0	0	0	0
100 μg/ml antisense XrelA	2	41	40	98	33	83	5	13	1	3	0	0	1	3
500 μg/ml x-relAΔ29	5	103	55	53	3	5	1	2	28	51	10	18	13	23
250 μg/ml x-relAΔ29	3	63	37	59	2	5	1	3	12	32	8	22	14	38
100 μg/ml x-relAΔ29	2	49	33	67	2	6	3	9	20	61	8	24	0	0
50 μg/ml x-relAΔ29	1	25	16	64	12	75	2	13	0	0	2	13	0	0
500 μg/ml uncapped x-relAΔ29	5	90	77	86	66	86	11	14	0	0	0	0	0	0

defect in mammals). With *Xenopus* this is a common artefact seen in many kinds of experiments and in our case it is certainly a non-specific artefact. Except where exogastrulation occurs, even the most affected embryos in this class gastrulate apparently normally, as judged by external criteria. At the equivalent of stage 30 they have little external evidence of a dorsal axis, head or tail; indeed superficially they are not unlike UV ventralised embryos at this stage (Scharf and Gerhart, 1980). They also lack visible segmentation of the somites. However, at later stages the existence of a nervous system is suggested by the appearance of two longitudinal stripes of melanocytes and histological examination shows that the central nervous system is indeed present, but in cross section it is small and poorly organised. However, neural differentiation is normal to the extent that it stains positively with 2G9, a monoclonal antibody marker of neural tissue (Jones and Woodland, 1989). The skeletal muscle blocks are roughly normal in cross sectional size, but they are very disorganised and there is no evidence of segmentation. They stain with the monoclonal antibody muscle marker B4 (Jones and Woodland, 1987). By contrast the notochord is much better organised and, although it is somewhat larger than usual, this is probably because the embryo does not elongate as normal, rather than because there is a great increase in total notochord tissue.

The least affected embryos typically have a kink in the mid-axial region. This is associated with locally disrupted segmentation and a small, poorly organised spinal cord in the mid-body. The relative amounts of other dorsal tissues is not noticeably disturbed and the notochord is normal.

Between the extremes there is a continuity of abnormality in which the head is reduced in size and then absent, and finally the tail is also absent. In all embryos the affected regions are characterised by disruption of tissue organisation, absence of, or poor segmentation, and reduction in the size of the nervous system. A very frequent additional abnormality in moderately affected embryos is thickening of the epidermis into warty patches, which in sections appear as multi-layered epidermis, staining positively with the epidermal marker 2F7 (Fig. 1J,K) (Jones and Woodland, 1986). Later these areas may proliferate into veil-like structures, reminiscent of tail fins (Fig. 1L).

Table 3
Effect of high XrelA1 levels on nucleic acid synthesis in embryos

	% total injected radioactivity		
	[^{3}H]Thymidine incorporation	[^{3}H]Uridine incorporation into total RNA	Proportion of total [^{3}H]RNA as poly(A)$^+$ RNA
Control embryos	13.6	2.6	4.1
XrelA mRNA (500 μg/ml)	9.5	1.8	7.8

Incorporation was measured after injecting blocked embryos at stage 10.5 with 50 nl [^{3}H]nucleoside at 10 mCi/ml. Total nucleic acids were phenol extracted as described in Section 2. Duplicate samples were acid precipitated onto glass fibre filters to give total [^{3}H]thymidine and [^{3}H]uridine injected; the total incorporation was expressed as a percentage of this. Similar duplicate aliquots were dried directly onto filters to determine total radioactivity. To measure the poly(A)$^+$ RNA, the remaining [^{3}H]uridine-labelled total nucleic acid samples above were ethanol precipitated and redissolved in 0.9 ml hybridisation buffer and shaken for 4 h with 1 mg oligo dT cellulose. The cellulose was pelleted and washed 2× with standard saline citrate (SSC) before eluting with distilled water at 100°C. The cellulose was pelleted and the radioactivity in the supernatant determined by scintillation counting.

3.3. Morphological effects of injection of XrelA1 mutants Δ29, Δ222

We previously showed that mRNA from *XrelA1Δ 29pSP64T*, abbreviated here to Δ29 mRNA, stimulated κB-dependent transcription, but to a lesser extent than the wild-type RNA (Richardson et al., 1994). At high levels it also produces the early block to gastrulation, but more must be injected than of the wild-type transcript. At lower levels it produced abnormalities that were indistinguishable from those produced by the wild-type (Tables 1A, 2). Δ29 lacks only the 29 C-terminal amino acids of the transcriptional activation domain, and produces somewhat reduced transcriptional activation (Richardson et al., 1994), as to a greater extent does a similar deletion of mammalian RelA (Schmitz and Baeuerle, 1991). Thus these results are consistent with the hypothesis that the gastrulation block and later abnormalities depend on the functioning of the activation domain.

Δ222 functions in DNA binding and dimerisation, but lacks all of the activation domain and it did not stimulate κB-dependent transcription, nor inhibit transcription of other promoters tested. On the other hand it suppressed transcriptional activation by injected wild-type XrelA1 mRNA (Richardson et al., 1994). It is therefore reasonable to propose that it would interfere with any endogenous p65 protein that might be present and active.

Δ222 mRNA at high levels did not arrest gastrulation, suggesting that this block depends absolutely on the presence of the activation domain. At the very highest levels of injected mRNA it produced a different, almost opposite, abnormality in gastrulation. The invagination of the posterior tissues was more complete than usual, so the yolk plug was not visible in the entrance of the terminal blastopore, as is usually the case (Fig. 1C). In later development such embryos show little development of the extreme posterior tissues, and an open, enlarged cloaca (Fig. 1I; Table 4). In the posterior part of the embryo segmentation is disrupted (Fig. 2). In the least affected embryos the main abnormality was an enlarged cloaca; in the most extreme the head is poorly formed or absent, as well as the tail.

3.4. Effects of XrelA1 and Δ222 on internal structure

We have made a histological examination of embryos that are moderately and slightly affected by injection of mRNA encoding wild-type XrelA1 (i.e. those embryos not blocked in gastrulation; Fig. 3) and also the Δ29 derivative, which gives phenotypes similar to the wild-type. The histology produced by the two messages is indistinguishable. In embryos in which head and tail structures are absent, the main classes of mesodermal tissues of the mid axis are present in roughly the usual amounts and with the appropriate dorso-ventral distribution. However the tissues are very disorganised and segmentation is not apparent. The most normal tissue is the notochord. The nervous system is present, but again is very poorly organised and its cellular part is thin compared to the size of the central cavity. In slightly less affected embryos the head contains placodal structures such as ear vesicles, but the nervous system is abnormally organised. As in the more severely affected embryos, in the mid-axis the notochord is again fairly normal (Fig. 3E,F), but myotomes are extremely disorganised, as is the lateral plate. The central nervous system is very small with a thin cylinder of cells. Disorganisation of the epidermis is also seen, with veil-like extensions as well as thickenings. Staining with the epidermal marker 2F7 (Jones and Woodland, 1986) shows that the cells are truly epidermal (Fig. 1J–L), i.e. it is tissue organisation rather than cell differentiation that is affected. This is also true of the myotomes which stain appropriately with the muscle marker (B4). In the least affected embryos, whose main external abnormality is a kink in the mid axis, the main internal abnormalities are a reduced, poorly organised central nervous system and disorganised somites in this region.

Abnormalities produced by over-expression of Δ222 are shown in Fig. 3G–I. This was an embryo of the type shown in Fig. 1I. The anterior regions are relatively nor-

Table 4
Injection of XrelAΔ222 mRNA

RNA sample	No. exp.	No. injected	No. reaching tailbud		Out of those reaching tail bud													
					Normal		Head good, tail poor		Head poor, tail good		Head poor, tail poor or absent		No head or tail		Poor axis		Spina bifida	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
XrelAΔ222																		
500 μg/ml	2	41	28	68	1	4	13	46	0	0	5	18	6	21	0	0	4	14
250 μg/ml	3	59	53	90	5*	9	25	47	0	0	18	34	1	2	1	2	4	8
125 μg/ml	2	40	36	90	24*	67	3	8	3	8	5	14	0	0	1	3	0	0
50 μg/ml	2	39	32	82	21	66	1	3	3	9	4	13	2	6	0	0	1	3
25 μg/ml	1	20	17	85	12	71	0	0	2	12	2	12	0	0	1	6	0	0
500 μg/ml uncapped	2	40	38	95	36	95	1	3	1	3	0	0	0	0	0	0	0	0

mal, but posteriorly the somites are disorganised and the nervous system is abnormal. The disruption of the somites in posterior regions is revealed in moderately affected embryos by wholemount antibody staining of the muscles with B4 and confocal optical sectioning (Fig. 2).

3.5. *Effects on endogenous gene expression of injection of XrelA1 and Δ222*

We have examined the levels of a number of mRNAs which are known, or strongly believed to have developmentally important roles. These are *Xbra, Goosecoid, Xsna, Pintallavis, Xwnt8,* and *Noggin* (Fig. 4). The high level of XrelA1, which blocks development at stage 10, substantially reduced the levels of all these genes except *Pintallavis.* Although in some cases lower concentrations of XrelA1 gave reduced amounts of these transcripts in Fig. 4, these reductions were not reproducible. In no instance was there a significant stimulation. This

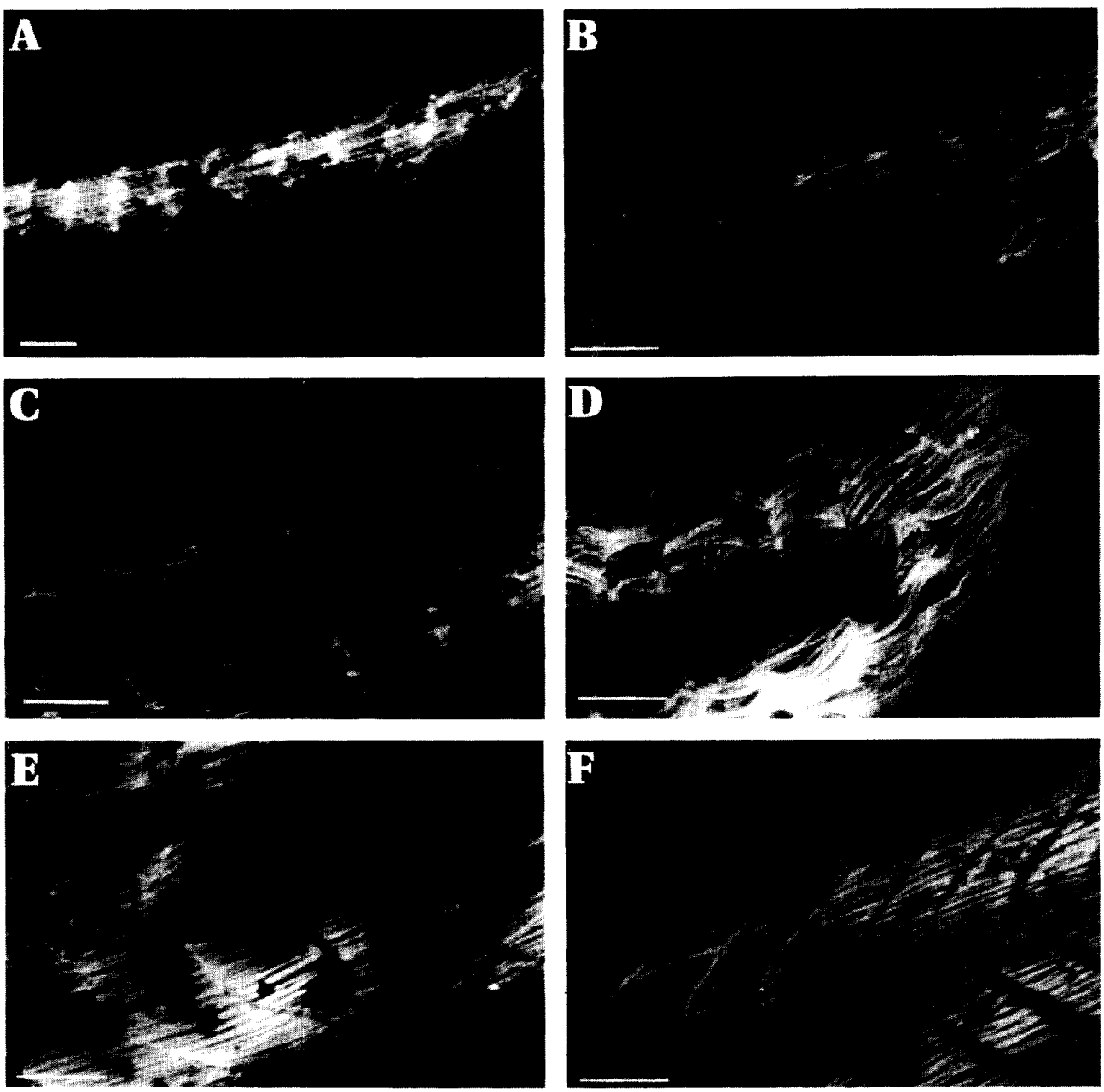

Fig. 2. Myotomes in mild to moderately affected embryos injected with capped Δ222 mRNA. Wholemount embryos were stained with the monoclonal antibody muscle marker B4 and optically sectioned by confocal microscopy. The embryos were abnormal in the very posterior regions (as in Fig. 1I) and the regions shown are just anterior to this. (A,B) Myotomes on two sides of an embryo injected with 10 ng mRNA; (B) is much more disrupted than (A). The different effect is presumably related to the fact that separate bilateral injections were made at the two-cell stage. (C,D) Similar views of a second embryo. (E) One side of a less affected embryo injected with 5 ng Δ222 mRNA. (F) Control injected with 10 ng uncapped Δ222 mRNA. The scale bars are 100 μm.

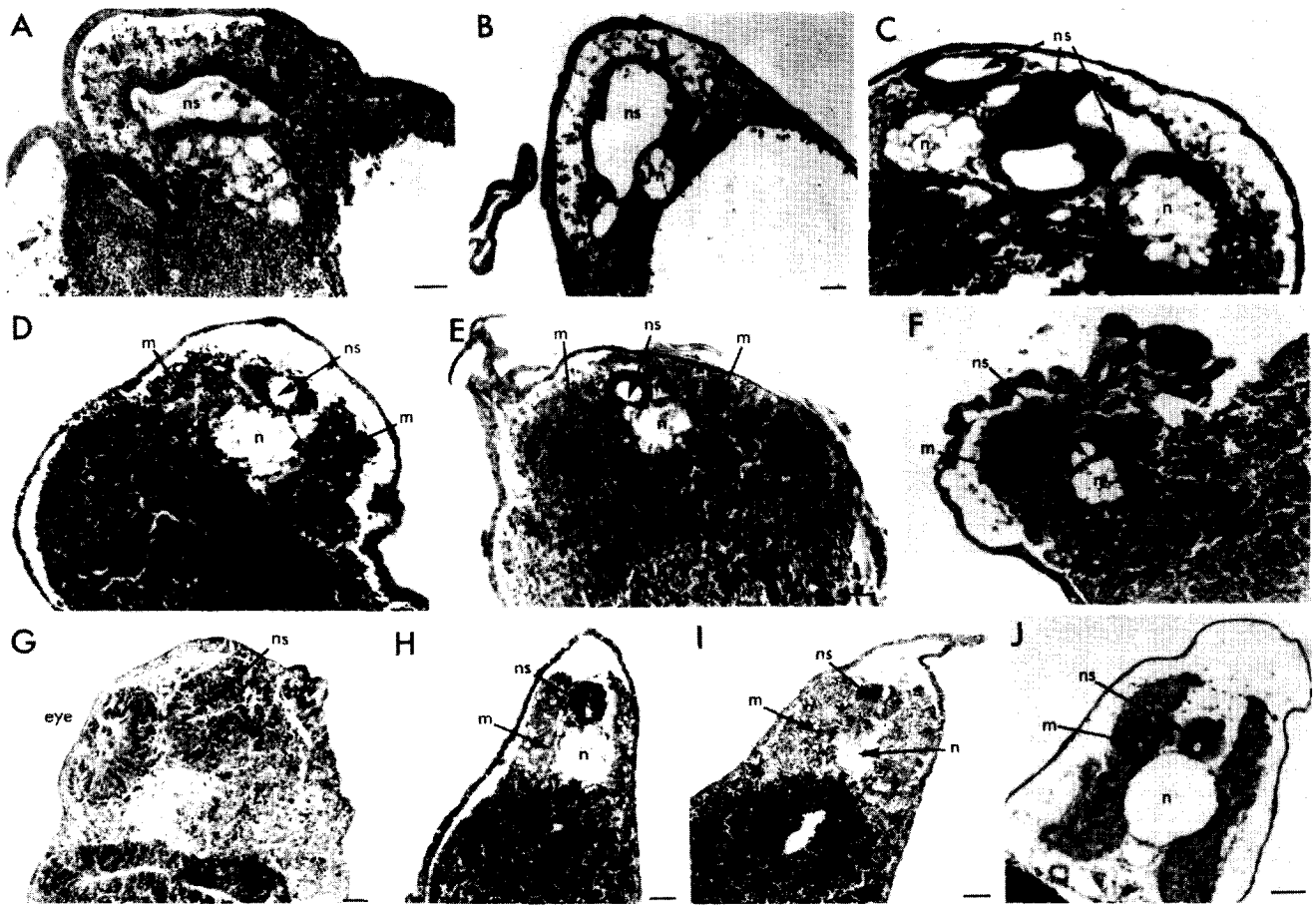

Fig. 3. Histological analysis of capped mRNA-injected embryos. (A) Extreme stage 37 embryo injected with 5 ng XrelA1 mRNA. Such an embryo has no externally discernible head or tail structures. The section is in anterior regions and small dark melanocytes may be seen (cf. Fig 1D). (B) Ear vesicle region of a slightly less affected embryo than in (A). (C–E) Stage 37 embryo injected with 10 ng Δ29 mRNA. (C) Anterior region; good notochord is seen as well as extensive, but abnormal nervous system. The axis was twisted, accounting for the appearance of axial tissues twice. (D) Anterior somitic region, showing relatively good neural and somite organisation. (E) Mid-axis showing the very poorly organised nervous system and somites, as well as extensions of the epidermis; in contrast the notochord is relatively normal. (F) Similar abnormalities in the mid axis of another embryo. (G–I) Moderately affected stage 37 embryo injected with 10 ng Δ222 mRNA. (G) Head region showing normal eye and extensive, but poorly organised, nervous tissue. (H) Normal mid-axis region. (I) Posterior region, showing disrupted somites and poor delineation of the notochord from the somites, but the central nervous system is more normal than for wild-type XrelA injections. (J) Normal mid-axis region of a stage 42 embryo injected with 10 ng anti-sense mRNA. Abbreviations: m, muscle tissue; n, notochord; ns, central nervous system. The scale bar represents 50 μm.

was true for the other markers (not shown). All of these conclusions also apply to *HoxB9* (*Xlhbox6*) and *Evx1* (*Xhox3*), detected at later stages, except that neither were significantly inhibited by the high XrelA1 injections.

Fig. 5 shows the effect of the dominant negative clone Δ222 on these genes. In no case is there a significant stimulation. Taken together the data on the wild-type and mutant XrelA1 indicate that none of the genes discussed above is under the direct control of XrelA1.

In situ hybridisation was used to analyse the spatial distribution of several transcripts (Fig. 5). In mid-gastrulation, low to moderate amounts of XrelA1 do not affect Xbra mRNA. At high levels the little that can be detected is located in a small region that may correspond to the dorsal lip. We cannot be sure, because albino embryos were used for the in situs. Even so the amount seen is substantially less than would be seen there in controls, and it is too little to see in the photograph.The distribution of *Krox-20* mRNA in later embryos that were affected enough to have little external evidence of head structure is illustrated in Fig. 5C. Expression in rhombomeres three and five is normal, but the branchial arch expression is absent. Even the rhombomere expression is not apparent in the most abnormal embryos. The expression of *Xtwi* was also present but was highly disorganised (data not shown).

3.6. Rescue of the XrelA1 gastrulation phenotype by Δ222

Both wild-type XrelA1 and Δ222 have strong biological effects so one is unlikely to be able to completely rescue the other. A complete rescue also depends on the heterodimer between the two having no biological activity. Table 5 shows that Δ222 relieves the block to gastrulation produced by XrelA1. The resulting embryos develop with

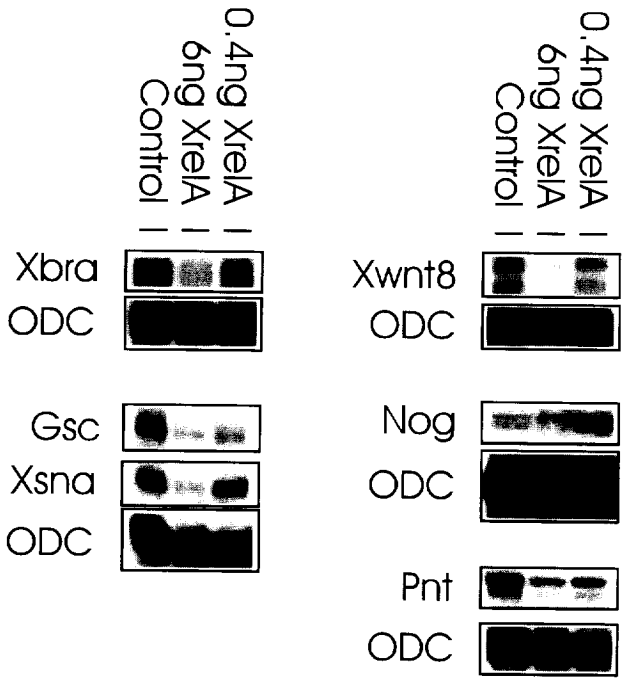

Fig. 4. RNase protection analysis of mRNAs in stage 10.5 gastrulae. The amount of XrelA1 RNA injected is shown above the tracks. Controls with an ornithine decarboxylase (ODC) probe were performed in the same protection assay as each of the experimental probes, and the result is shown under the bands for the gene product under test. Goosecoid, Xsna and ODC were analysed in a three probe mixture.

the abnormalities associated with Δ222 (e.g. the warty epidermal structures associated with XrelA1 are absent and the cloaca is enlarged). Table 5 also shows that co-expression of mammalian p50 relieves the gastrulation

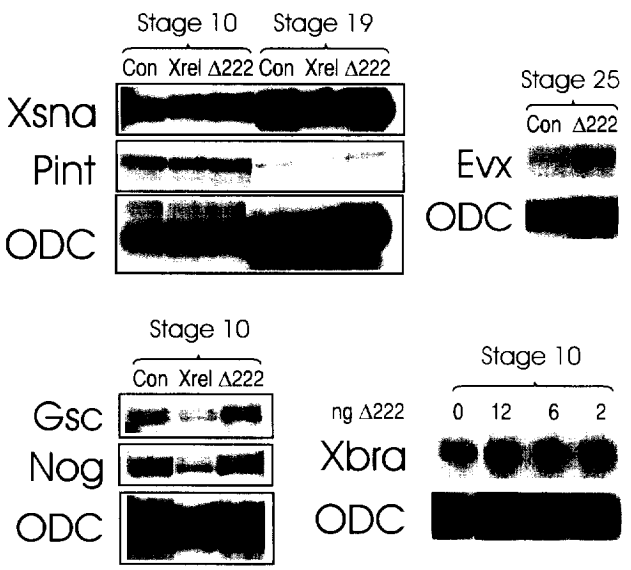

Fig. 5. RNase protection analysis of embryos injected with the dominant negative construct Δ222. 6 ng of Δ222 and 0.4 ng XrelA was injected except for the Xbra experiment, where the amount injected was as shown. The stages at which the RNA was extracted are shown.

Table 5
Rescue of the XrelA gastrulation block with Δ222

Injected mRNA	No. injected embryos cleaving normally	% embryos blocked at stage 10
1 ng XrelA	21	90
1 ng XrelA + 10 ng Δ222	20	0
10 ng Δ222	17	0
1 ng p50	16	0
1 ng p50 + 500 pg XrelA	13	0
500 pg XrelA	17	100

block. Subsequently these embryos develop the same abnormalities as with XrelA1 alone.

4. Discussion

Two kinds of phenotype were generated by over-expression of wild-type XrelA1: high levels halted gastrulation after apparently normal formation of the dorsal lip, lower levels disorganised tissue structure. It is not clear that these two results are really related, since the former may involve non-physiological inhibition of transcription. We previously found that a viral thymidine kinase promoter was drastically inhibited by high levels of XrelA1 (Richardson et al., 1994), suggesting that some kind of general interference with transcription (squelching) might have occurred. In turn such an effect could lead to the block to gastrulation. However, we also found that transcription from an injected N-CAM promoter was unaffected and we show here that a substantial amount of poly(A)$^+$ RNA synthesis occurred after developmental arrest of the embryos. This shows that not all genes are inhibited, and this is supported by our measurements of endogenous transcript levels (see below). It has long been known that actinomycin D and α-amanitin arrest development at the late blastula stage (Brachet and Denis, 1963; Wallace and Elsdale, 1963; Newport and Kirschner, 1982), but with these inhibitors the dorsal lip does not form at all, as it always does in our experiments. Thus it appears that XrelA1 does not inhibit all transcription. Lastly, we previously noted that the transcription of the κB-dependent HIV promoter was not stimulated at all by high XrelA1 levels, although it is strongly stimulated by low levels. This opens the possibility that any endogenous κB-dependent promoters that were being driven by this control element would be specifically inhibited by high XrelA1, i.e. it would act, at least in part, in a dominant negative fashion.

We observed a 30% reduction in thymidine incorporation after gastrulation was blocked. Reduced cell number could be one reason why the RNA synthesis was reduced. A reduction in cell division per se would not have blocked gastrulation or led to considerable developmental abnormalities. Cooke (1973) blocked cell division and

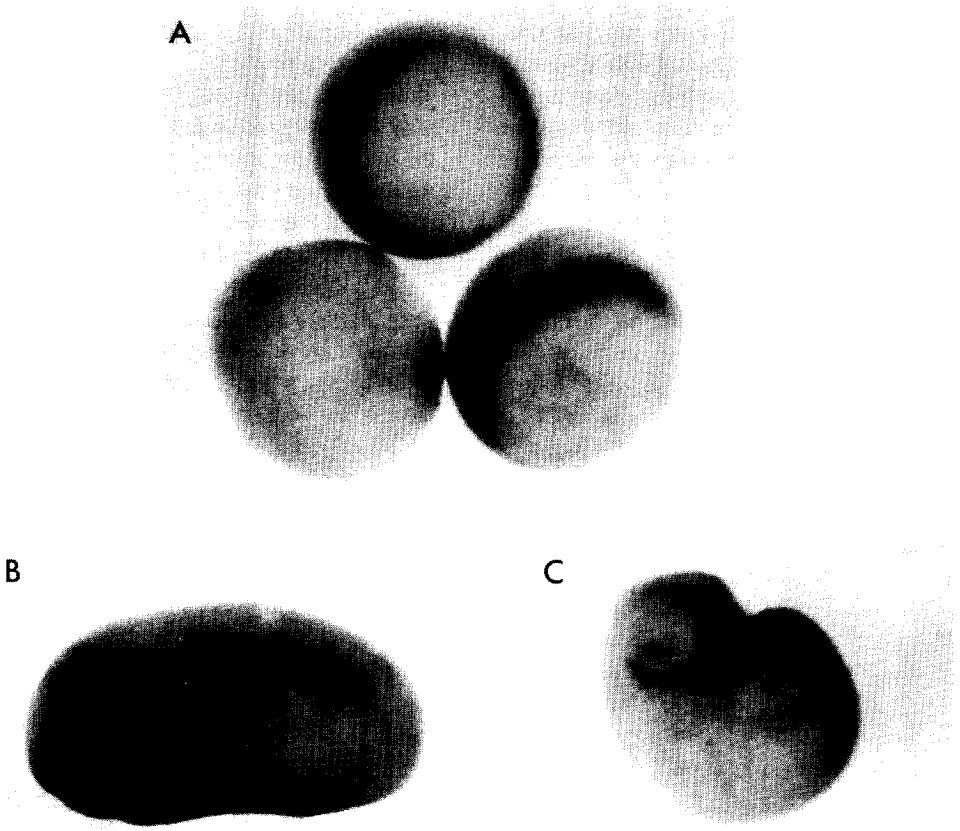

Fig. 6. Whole-mount in situ hybridisations to Xbra and Krox-20 RNA. (A) Embryos probed for Xbra mRNA distribution at stage 10.5: a, control; b, injected with 5 ng XrelA mRNA; c, injected with 10 ng Δ222 mRNA. (B,C) Stage 21 embryos probed for Krox-20 RNA: a, control; b, injected with 0.5 ng XrelA1 mRNA.

Harris and Hartenstein (1991) blocked DNA synthesis in early gastrulae and showed that development through neurulation was remarkably normal in the absence of any cell division.

We have measured the levels of a number of developmentally important genes that are transcribed around the mid-blastula transition (MBT) and gastrulation. These include *Xbra, Goosecoid, Xsna, Pintallavis, Xwnt8, Noggin, HoxB9* (*Xlhbox6*) and *Evx1* (*Xhox3*). All except the last two were depressed by high levels of XrelA1, but, particularly because development is blocked, this depression could be a relatively distant consequence to changes in development produced by XrelA1. Certainly none was stimulated, which suggests that none has promoters directly driven by XrelA1. It is interesting that an apparently normal dorsal lip appears, even when there is very little *Xbra* or *Goosecoid* expression. This might be expected for *Xbra* because loss-of-function *Brachyury* mutant mouse embryos mouse are deficient in posterior, but possess anterior mesoderm (Chesley, 1935; Gluecksohn-Schoenheimer, 1944; Beddington et al., 1992). By analogy with mice, the lack of lateral and ventral *Xbra* expression would be a sufficient reason for the failure of the blastopore lip to appear in these regions. The *Goosecoid* result is more unexpected, given that *Goosecoid* mRNA is localised to the organiser and when injected it leads the formation of a new organiser (Cho et al., 1991). Thus either the function of *Goosecoid* is redundant in formation of the dorsal lip, or much reduced amounts of Goosecoid protein are sufficient to form it.

Effects of lower dosages of XrelA involve tissue organisation, rather than the quantity of tissues in the mid-axis. This statement primarily concerns the dorsal tissues, mainly because they are larger and more highly structured than embryonic ventral tissues, and therefore such effects are more easily scored (however, the ventral marker *Xwnt8* was not particularly affected, so there is probably no dramatic effect on the amount of ventral tissue). The central nervous system is reduced in size and poorly structured, especially in the mid-axis. The somites are also very poorly organised and lack discernible segments, but the notochord is relatively much more normal. In badly affected embryos head and tail development is not discernible, whereas less abnormal embryos are mainly affected in the mid-body; the axis is kinked, somites are disrupted and the CNS is poorly developed. Thus overexpression does not obviously lead to a reallocation of cells to different dorso-ventral tissue types, rather there is

an effect on organisation, e.g. segmentation. It could be argued that over-expression is most likely to affect regions where a gene product is not normally expressed. According to this hypothesis, since the least affected embryos are abnormal in the mid-axis, a role in the termini can be postulated. This idea is supported by the dominant negative clone *Δ222* (see below).

Transcripts of the clone encoding a protein truncated of the 29 C-terminal amino acids have the same phenotypic effects as the wild-type, but relatively higher doses are needed to generate abnormalities of the same degree of severity. This is consistent with our observation that this protein is also less efficient than the wild-type at stimulating κB-dependent transcription of an injected gene (Richardson et al., 1994); as is also true of mammalian RelA, although here the difference was more pronounced (Schmitz and Baeuerle, 1991).

The clone encoding a protein deleted of its 222 C-terminal amino acids does not stimulate κB-dependent transcription of a model gene, but rather inhibits the effect of the wild-type in such stimulation. However this occurs only at the highest mRNA injection levels (Richardson et al., 1994). At no concentration did this clone block gastrulation. The typical moderate severity phenotype was for the final stages of gastrulation to be modified, resulting in a pit-like final blastopore and ultimately an enlarged cloaca. Subsequently the tail failed to develop properly and segmentation was disrupted over up to 30% of the caudal part of the antero-posterior axis. In more severely affected embryos the head was abnormal or poorly developed. These data, like those for the wild-type clone, are consistent with a role for *XrelA* in antero-posterior patterning, most importantly in the posterior regions and to a lesser extent in the head.

Controls are critical for all of the phenotypic effects described above. We have used uncapped and anti-sense transcripts from the same DNA preparations under study. Neither make proteins in the embryo, but both should contain the same kinds of impurities as might be present in the capped mRNA preparations. In no case were there abnormalities of the kind described for the capped mRNAs. Any abnormalities were at low frequencies and were typical artefacts (like exogastrulation) of any experiments on *Xenopus* embryos. One clone also provides controls for the others. Thus Δ222, which is expressed as protein more efficiently than the wild-type, did not produce arrest of gastrulation, indeed its effects at high concentration could be regarded as almost the opposite of gastrulation arrest. This interference with posterior development was not seen with the other clones. Lastly we attempted a rescue of XrelA1 by Δ222. The block to gastrulation was completely removed, but the embryos show the characteristics of Δ222 over-expression. While this could be caused by failure to find exactly the right level of the two mRNAs it is much more likely that the XrelA1/Δ222 dimer does not transactivate and therefore functions like Δ222. This would be hard to test. We also showed that mammalian p50, which does not produce a phenotype, and which scarcely transactivates the HIV LTR (Sutherland and Woodland, unpublished), rescues the block to gastrulation, but does not rescue the later defects associated with XrelA1 over-expression. Since p50/XrelA1 dimers transactivate efficiently, the latter is not surprising. Taken together these results indicate that the gastrulation block is not caused by trivial artefacts and depends on a molecule having two XrelA1 Rel homology regions and two activation domains.

In a search for targets of XrelA1, as well as to define defects in more molecular terms, we have measured the levels of a number of transcripts at moderate and low levels of XrelA1 overexpression (see above). These are all markers of major portions of the dorso-ventral or antero-posterior axes, so the fact that none are radically decreased or increased supports the view that XrelA1 does not control the allocation of tissues to these major portions of the body plan. There are provisos to this interpretation, arising from the limitations of the available markers; for example no very posterior dorsal marker was used; HoxB9 is posterior only in the sense that the whole post-cranial nervous system is posterior (The effects of Δ222 on morphology described above are more posterior than this). The chief effect of low levels of XrelA1 was on morphological organisation within the tissues and this was also seen when in situ hybridisation was performed using *Krox-20* and *Xtwist* probes. The kinds of target molecule that could produce such effects might be cell adhesion proteins or those concerned with producing and adhering to the extra-cellular matrix. Such molecules are known targets of homeotic genes in *Drosophila* (Gould and White, 1992) and it is known that overexpression of the homeotic gene *Xhox-1A* disrupts segmentation in *Xenopus* (Harvey and Melton, 1988).

In conclusion, we believe that it is unlikely that *XrelA1* is performing the same pivotal role in dorso-ventral axis formation that its homologue *dorsal* is in *Drosophila*. Taken together the data (particularly from the interference clone *Δ222)* suggest that it has a role in the patterning of the termini, especially in the posterior region. A *proviso* to this conclusion is that *Δ222* probably interferes with XrelA by titrating out κB sites (Richardson et al., 1994) and it could therefore be revealing the function of another gene product, probably also a *rel*/NF-κB/*dorsal* family member, that binds to these sites. A role for *Xenopus dorsal* homologues in terminal patterning is in fact not so unexpected as one might at first imagine. Although in *Drosophila* the role of *dorsal* is most obvious in forming the dorso-ventral axis under the control of the Toll ligand, *dorsal* is in fact under *torso* control at the termini of the *Drosophila* embryo (Casanova, 1991; Rusch and Levine, 1994). Perhaps it is this terminal role that has been conserved in vertebrates.

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