The development of an assay to detect mRNAs that affect early development

H. R. WOODLAND and E. A. JONES
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Summary
We have constructed an assay to identify developmental effects of injected RNA molecules. The RNA is injected into the animal pole region of a 2- to 8-cell embryo. At the blastula stage, the animal cap is removed and its development in isolation tested. In controls, only epidermis is produced, but several of the injected RNA preparations, though not all, also form dorsal and ventral mesoderm and nervous tissue. This assay should be suitable for selecting cDNA clones complementary to mRNAs that direct development.

Key words: mRNA, Xenopus, assay, cell signalling.

Introduction
Recent studies of Drosophila have underlined the necessity for genetic analysis in understanding development. However, in vertebrates it has not been possible to apply standard diploid, meiotic genetics to the identification of the genes involved. A viable alternative approach might be to design functional assays for mRNAs that can produce observable perturbations in development. These assays could then be used to clone cDNAs complementary to the RNAs responsible for these effects.

We have developed one such approach based on the proposition that mRNA populations might change the fate of the daughters of blastomeres into which they are injected. This assay might also lead to changes in the fate of surrounding cells if the injected mRNAs encode functions related to cell signalling.

Materials and methods

Embryo manipulations and histology
Xenopus embryos were obtained from natural matings or by in vitro fertilization and injected under 5% Ficoll by standard procedures (Wilson et al. 1986). RNA was injected at 1–2 mg ml⁻¹ in 1 mM-EDTA, 10 mM-Tris–HCl, pH 7.6. Embryos were allowed to develop to stage 9 in modified Barth’s solution (Gurdon, 1977), usually overnight at 15°C, and the animal caps removed. These caps were cultured until control embryos reached stage 31–36, fixed, embedded, sectioned and stained with antibodies as described previously (Jones & Woodland, 1986).

Biological materials
Monoclonal antibodies were used to identify differentiated muscle, nervous system and epidermis. Striated muscle was identified by 5A3.B4 (Jones & Woodland, 1987b). The antigen recognized by this antibody first appears at early tailbud. Differentiated nervous system was identified by 2G9, an antibody raised against adult Xenopus brain. The antigen recognized by this antibody first appears at stage 21 and is entirely specific to central nervous system (our unpublished data). The epidermal marker used in these studies was 2F7.C7 (Jones, 1985; Jones & Woodland, 1986). All monoclonal binding was detected with fluorescein or rhodamine-conjugated rabbit anti-mouse IgG (Miles Ltd).

XTC-2 cells (Pudney, Varma & Leake, 1973) and XP cells were maintained at 25°C in 67% Leibowitz L-15 medium or GMEM, (GIBCO), respectively, supplemented with 10% fetal calf serum and penicillin and streptomycin.

Preparation of RNA
RNA was prepared from embryos, eggs and ovary by a scaled up version of the procedure of Wilson, Cross & Woodland (1986). Briefly, this involved proteinase digestion, phenol/chloroform extraction and ethanol precipitation. Volumes were increased in proportion to the number of embryos used. This procedure was also used to prepare RNA from cultured XTC-2 cells. The cells were
washed three times with phosphate-buffered saline in their culture bottles, then lysed in situ with 5 ml of the same homogenization buffer used for embryos. The subsequent procedure was as for embryos.

Some RNA preparations from cultured cells were made with a guanidinium iso-thiocyanate caesium/chloride procedure (Maniatis, Fritsch & Sambrook, 1982).

Polyadenylated RNA was made by oligo dT-cellulose chromatography, according to Maniatis et al. (1982), except that LiCl was substituted for NaCl in the buffers. Polyadenylated RNA from all sources was translated in vitro using rabbit reticulocyte lysate or a wheat germ system. Every one generates a full range of molecular weight products. The products of translation were run on 15% or 18% SDS–PAGE and visualized by autoradiography.

RNA was fractionated on sucrose gradients according to Woodland (1974).

Results

Principle of the embryo assay

Isolated fragments of the blastula animal cap develop into epidermis (Holtfreter & Hamburger, 1955; Grunz, 1977; Slack, 1984; Jones & Woodland, 1986). We have investigated whether the range of tissues that these cells form can be expanded by injecting a population of mRNAs into the animal region of the egg or very early embryo. The injected cell and its daughters could show changed developmental potency, or the products of injected cells could directly influence neighbours. The detailed interpretation of these experiments is therefore complex (see Discussion), but the assay for mRNA function has the advantage that its design is straightforward. The animal pole of a 2- to 4-cell embryo is injected with poly(A)+ RNA. At the late blastula stage the animal cap is removed, cultured to stage 30–36, fixed, sectioned and analysed for the appearance of epidermis, mesoderm and neural tissue. Polyadenylated RNAs from a number of sources have been used, but in an attempt to simplify the expected results, we have concentrated on XTC-2 cell RNA. All the poly(A)+ mRNAs used in this study were translated in vitro using either wheat germ or reticulocyte systems. In all cases, including those with no biological activity, the RNAs gave high molecular weight translation products. These cultured Xenopus cells secrete a potent inducer of dorsal and ventral mesoderm (Smith, 1987). Their RNA would therefore be expected to confer on an injected animal cap cell the property of inducing its neighbours (or even itself) to form mesoderm.

Effects of injecting XTC cell RNA

Table 1 shows the pooled results from seven different experiments carried out with four different mRNA preparations from the XTC-2 cell line, injected into early embryos and dissected as just described. The embryos ranged from stage 2 to stage 5 at the time of injection, and were all dissected at stage 9 to yield animal cap explants. More than 55% of the caps analysed showed clear mesodermal structure. This ranged from lateral plate and loosely packed mesenchymal mesoderm, to highly organized striated muscle, and in one case notochord (Fig. 1C). In all instances, muscle was positively identified with the muscle-specific monoclonal 5A3.B4 (Jones & Woodland, 1987b) and notochord with the notochordal marker (Smith & Watt, 1985). In all, 20% of the explants contained fully differentiated muscle. If 2-cell embryos were used for injection, approximately 30% of the explants contained differentiated muscle.

Figs 1 and 2 show the morphology characteristic of the different classes of explants observed, and their staining with specific monoclonal antibodies to identify muscle, neural tissue and notochord. In cases where the morphology was less pronounced, explants were stained with the epidermal marker 2F7.C7. Previous studies have shown that ectodermal sandw iches containing mesoderm have the epidermal

Table 1. Analysis of XTC-2- and ovary mRNA-injected animal caps

<table>
<thead>
<tr>
<th>Source of mRNA</th>
<th>No. analysed</th>
<th>No. with mesoderm</th>
<th>% with mesoderm</th>
<th>No. with muscle</th>
<th>% with muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>XTC-2</td>
<td>97</td>
<td>54</td>
<td>56</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>XP</td>
<td>13</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xenopus ovary</td>
<td>15</td>
<td>11</td>
<td>73</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>Pea leaf</td>
<td>16</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Control uninjected</td>
<td>31</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Poly (A)+ mRNA from XTC-2 cells was injected into Xenopus embryos at stage 2-5. Animal caps were isolated at stage 9 and incubated until control embryos reached stage 30–36.
Fig. 2. Morphology of explants. Embryo explants were made as in Fig. 1. Injection of XTC-2 polyadenylated mRNA frequently resulted in explants with complex ventral mesodermal structure (A). These were often negative for the muscle and notochordal antibody markers (B). Bar, 75 μm.

staining pattern characteristic of whole embryos, i.e. only the outer layer of the explant expresses the antigen. In sandwiches or explants, lacking mesoderm, most internal cells are positive for 2F7.C7 (Jones & Woodland, 1986, 1987a). The former kind of staining is shown in those explants from RNA-injected embryos that show complex structure of a ventral mesodermal type (Fig. 1A). In the absence of ventral mesoderm molecular markers, this staining pattern is consistent with the presence of ventral mesoderm within these animal caps. As one kind of control, embryos were injected with mRNA from the XP cell line, which does not produce a mesoderm-inducing factor (unpublished observations). Only one explant out of the thirteen analysed contained ventral mesoderm and none contained muscle. Similarly, one explant out of the sixteen analysed after injection with pea leaf mRNA contained mesoderm. In this case, it did contain muscle. None of the 31 control uninjected explants contained muscle, although one did have ventral mesoderm. The small percentage of these various control explants that show mesodermal structure is consistent with dissection error.

To validate the effect shown by total poly(A)+ RNA an attempt has been made to size fractionate the mRNA from XTC-2 responsible for the effect in mesoderm formation. Sucrose density gradient fractions were assayed for inducing ability after ethanol precipitation. Fig. 3A shows the absorbance profile of the gradient, its in vitro translation and the results of injecting the mRNA into 2-cell embryos. The latter results have been pooled from four different gradients, using two independent mRNA preparations, analysed in two injection experiments. Two peaks of mesoderm-inducing activity can be seen. One corresponds approximately to the major translatable mRNA peak. The other peak, representing the majority of the activity, resides near the bottom of the gradient. For the gradient actually shown in the absorbance and translation profiles, the mRNA was not heated before loading. There were small amounts of several proteins made from certain mRNAs that smeared down the gradient. In two other gradients the RNA was heated; there were no detectable in vitro translation products in the bottom fractions, but they still contained mesoderm-inducing activity. The injection results of all experiments are pooled in Fig. 3B. It is not yet clear whether the rapidly sedimenting activity was produced by large molecular species or aggregates.

Effect of embryonic RNAs
Preliminary results have also been obtained by injecting mRNAs prepared from different embryonic stages and from ovary, into early embryos. These results suggest that similar effects can be observed with mRNA from these sources. If ovary poly(A)+ mRNA is injected into stage 2–4 embryos, complex mesodermal structure was observed in 73% of cases (total 15) and 40% contained muscle. If early neurulæ are used as the source of mRNA, a small, but significant, proportion of the dissected explants contains differentiated nervous system as identified by a monoclonal marker 2G9 (our unpublished data). 44% of explants derived from embryos injected with early neurula mRNA (18 in total) had a complexity of structure not normally seen in ectodermal explants and 16.6% stained for nervous system (Fig. 1D).
Discussion

mRNA assays involving blastomere injection

In these assays, blastomeres are injected in the animal cap region and the later development of isolated animal caps is followed. Normally these explants would form only epidermis, but after RNA injection we find mesodermal tissues and nervous system. The procedure has the advantage that it is relatively simple to perform. However, its interpretation could be complex. The specification of the descendants of the injected cell could be altered if individual mRNAs were, or encoded, embryonic determinants. Alternatively, the injected cell could affect the development of its neighbours. This could be directly because the injected RNA translated into cell-interaction molecules, or indirectly because the injected cell differentiated into a type of cell that produces such molecules. Lineage labelling could help with this interpretation, but it would be unlikely to be definitive. This is because the injected cell will divide into many daughters which could interact with each other, and a cell making a type of signalling molecule which it would not normally produce, could change its own fate. Ultimately, the interpretation will depend on identifying the RNA concerned. In this paper, we have been concerned with establishing only if the assay is able to generate useful results.

Cells injected with RNA from XTC-2 cells should secrete mesoderm inducer and, therefore, they should induce their neighbours or themselves to become mesoderm. We find that explants from such
injected embryos form mesoderm at high frequency and that this often includes muscle and occasionally notochord. This effect is only very rarely seen with RNA from XP cells. These cells do not induce mesoderm when in combination with competent animal cap cells (unpublished data). It was also seen once in 16 cases with pea leaf RNA. Presumably these represent experimental error, but it is impossible to be quite certain a priori that any given RNA will be devoid of biological activity. For example, plant lectins can influence cell differentiation. Nevertheless these low levels support the view that the result with XTC-2 RNA is meaningful.

XTC-2 poly(A) + RNA fractionated on sucrose gradients yields two peaks of activity, one a little smaller than 18S and the other apparently very large. The fact that there are graded peaks again supports the idea that particular RNA species generate the effect. However, the apparently very high molecular weight RNA could be aggregated. Polyadenylated RNA from stages between oocyte and neurula all produce mesodermal induction. This effect could certainly be produced by mesoderm-inducing mRNAs. Mesoderm-induction signals exist before the general activation of transcription in the blastula (Jones & Woodland, 1987b) so one would expect the mRNA responsible to be present in the oocyte. There is also the capacity to induce mesoderm in the tail region of the neurula (our unpublished data), so the presence of this activity in neurula RNA is also likely. Although we favour the interpretation above, it is possible that oocyte or other RNAs are causing vegetal cells to develop in the animal region and hence to induce their neighbours to form mesoderm. It is extremely difficult to distinguish between these two possibilities experimentally. Clearly the next stage in analysing these effects will be to attempt to clone the mRNAs responsible.

We have also carried out preliminary experiments in an attempt to develop an assay based on the grafting of undetermined animal cap cells on to the surface of oocytes previously microinjected with embryonic mRNAs. We have found that oocytes grafted in this way can be cultured for several days. Preliminary experiments using neurula mRNAs suggest that this assay could be used to identify cell interaction molecules, for example, inducing substances, since in a small percentage of cases differentiated nervous system can be identified in the grafted ectoderm and, in a larger number of cases, complex morphology is observed (our unpublished data). This assay clearly has a potential use to identify cell interaction molecules, providing no negative-controlling elements for the active mRNAs are present in the oocyte. This situation does seem to be the case for mesoderm induction since XTC mRNA injected into oocytes which are subsequently grafted with ectoderm fails to generate mesoderm in the grafts. This is not unexpected since uninjected controls do not induce mesoderm. The signal to induce mesoderm in the embryo starts before new transcription and so, presumably, must be translated from a maternal transcript. The oocyte, therefore, must have a way of suppressing this activity. Injected mRNAs might be subject to similar controlling mechanisms.

The construction of assays such as these should permit cloning of mRNAs that control development. They could not be used to assay mRNAs hybridized to filter-bound DNA from pools of clones, as was done to clone interferon (Nagata et al. 1980). Alternatively, artificial mRNA could be synthesized from pools of cDNAs cloned in SP6 vectors, as was done to clone an interleukin-like factor (Noma et al. 1986).

This work was funded by the Medical Research Council and the Agriculture and Food Research Council. The authors acknowledge the clerical assistance of Mrs Len Schofield and the technical assistance of Andrea Haworth and John Ridley and the gift of pea leaf poly(A) + mRNA from Dr M. Hartley, University of Warwick.

References


(Accepted 21 September 1987)