The initiation of new gene transcription during *Xenopus* gastrulation requires immediately preceding protein synthesis

S. CASCIO and J. B. GURDON

Cancer Research Campaign, Molecular Embryology Research Group, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK

Summary

The incubation of *Xenopus* embryo fragments in cycloheximide at 5 or $10 \mu g \, ml^{-1}$ rapidly inhibits protein synthesis to 10 % or less of control levels. In most batches of embryos, treatment with cycloheximide for up to 1 h causes no obvious cellular damage and protein synthesis is fully restored to normal levels 5 h later. Transcript analysis with RNA probes shows that the inhibition of protein synthesis at late blastula or early gastrula stages completely suppresses the normal initiation of actin gene transcription at the mid–late gastrula stage. This applies to muscle-specific actin genes, whose transcription is initiated by induction, as well as to cytoskeletal actin genes not activated by induction. Two-dimensional gel protein analysis shows that cycloheximide irreversibly inhibits only 10 % of all genes normally expressed at a postneurula stage and that all of these are genes whose expression is normally initiated during or soon after gastrulation. Cycloheximide treatment causes a limited reduction of DNA synthesis, and no reduction of overall RNA synthesis. We conclude that the initiation of new gene transcription during gastrulation in *Xenopus* is dependent on the immediately preceding synthesis of certain proteins.

Key words: gene transcription, *Xenopus laevis*, gastrulation, protein synthesis, cycloheximide, actin gene.

Introduction

In *Xenopus* development some kinds of genes are first transcribed at the mid-blastula transition, 5 h after fertilization; these include 5S RNA, tRNA and some small nuclear RNAs (Brown & Littna, 1966; Woodland & Gurdon, 1968; Forbes, Kornberg & Kirschner, 1983), each of which is transcribed in all cells of an embryo. Several other genes start to be transcribed during gastrulation and these include the first genes to be expressed in some cells but not others, i.e. in a cell-type-specific way. Among these are muscle-specific actins (Mohun, Brennan, Dathan, Fairman & Gurdon, 1984) and epidermal cytokeratins (Jonas, Sargent & Dawid, 1985; Winkles, Sargent, Parry, Jonas & Dawid, 1985).

The mechanism by which transcription of the cardiac actin gene is initiated is particularly suited for more detailed analysis. This is because it can be activated as little as 7 h after animal and vegetal regions of a blastula have been placed in contact, even though neither part cultured on its own transcribes these genes (Gurdon, Fairman, Mohun & Brennan, 1985). Ideas on the events that may take place during the 7 h induction process fall into two classes. According to one view, preformed substances are mobilized by induction and proceed to activate muscle actin genes directly, without the need for any preceding RNA or protein synthesis. The alternative class of explanation supposes that an immediate effect of induction is to cause expression of an unknown gene or genes, and that it is these gene products that initiate actin gene transcription. These two concepts can be distinguished by reversibly inhibiting protein synthesis at various times during the induction process. The combination of a short induction period with an early and quantitative molecular assay makes it possible to analyse this example of gene activation in development in this way. Here we describe experiments which clearly support the second of the two concepts outlined above.

Materials and methods

Whole embryos, animal caps and conjugates

Normal blastulae, reared from fertilized eggs, were divided into animal, equatorial and vegetal regions, as described by Gurdon *et al.* (1985). When cultured in isolation, neither

animal nor vegetal regions initiate transcription of muscle-specific actin genes. But when placed in contact as a conjugate, some of the animal cells are induced by vegetal cells to activate their muscle-specific actin genes. Animal regions of a blastula are referred to as 'animal caps'. If they have been placed in conjugation with a vegetal region which is subsequently removed, they are referred to as induced animal caps. All living material has been cultured in modified Barth solution (Gurdon, 1977). The removal of vegetal cells from conjugates was carried out manually as described by Gurdon *et al.* (1985); it was not necessary to prove, in the present series of experiments, that every vegetal cell was removed.

Cycloheximide treatment

Cycloheximide (Sigma) was made up in modified Barth solution. At the end of the treatment period of 1 h, animal caps or conjugates were removed from cycloheximide, washed well in several changes of medium and cultured further. In every experiment, a small number of embryos or fragments were labelled with [^{35}S]methionine, to check that the cycloheximide treatment had been effective.

Labelling

Proteins were labelled by injecting animal caps with 20 nl [^{35}S]methionine (<800 Ci mmol^{-1}) at 10 mCi ml^{-1}. After injection, animal caps were incubated during the labelling period in [^{35}S]methionine at 1 mCi ml^{-1}. To measure DNA synthesis, animal caps were incubated for 2 h in [^{3}H]thymidine (25 Ci mmol^{-1}) at 0·83 mCi ml^{-1}. To determine acid insoluble incorporation, 5 μl of a homogenate was spotted onto a Whatman GFC filter. Filters were placed in 400 ml of ice-cold 10 % TCA, kept on ice for 30 min, then washed twice with distilled water, twice with 95 % ethanol and twice with acetone before being dried and counted.

Transcript analysis by nuclease protection

We have used exactly the same cDNA in an SP6 vector and the same procedures for preparing labelled RNA probe, RNase digestion and gel analysis of protected probe, as before (Gurdon *et al.* 1985). The recognition of muscle or cytoskeletal actin transcripts according to the size of the protected fragments is described in the legends to Figs 3 and 4.

Protein analysis by two-dimensional (2D) gel electrophoresis

Frozen samples in about 50 μl of buffer (2 % sodium dodecyl sulphate, 80 mM-Tris pH 6·8, 10 % glycerol and a trace of bromophenol blue) were sonicated for 10 s on an Ultrasonics Sonicator at its highest speed and microfuged for 10 min. The supernatant was then mixed with an equal volume of O'Farrell's lysis buffer and the two-dimensional analysis carried out according to O'Farrell (1975) except that double the recommended ampholine concentration was used in the isoelectric focusing gel. Each analysis contained no more than three animal caps, which would usually contain at least 200 000 TCA-precipitable counts. Autoradiographs of gels were usually for 1 week.

Poly(A)$^+$ RNA selection

A column of polyU sepharose (Pharmacia), 1×1 cm, was prepared in binding buffer (10 mM-Tris–HCl, pH 7·4, 100 mM-NaCl, 10 mM-EDTA). The column was washed with 5 ml of elution buffer (10 mM-Tris–HCl, pH 7·4, 100 mM-NaCl, 10 mM-EDTA, 90 % deionized formamide) and then washed again with 10 ml of binding buffer. Each sample (about 50 μg RNA from 15 conjugates) was loaded in 2 ml of binding buffer and the flow-through material reloaded twice more. Poly(A)$^-$ fractions were then collected from 10 ml of binding buffer, all labelled RNA coming out in the first 3 ml. Then 6 ml of elution buffer was passed through, all poly(A)$^+$ labelled RNA again appearing in the next 3 ml. Part of each fraction was counted in a scintillation counter; the remaining parts of fractions containing RNA were pooled, precipitated and counted. Using exactly the same column materials, procedures and buffers, it has been established in this laboratory that almost none of the flow-through poly(A)$^-$ RNA binds to a new column on being reloaded.

Results

Cycloheximide inhibits protein synthesis in Xenopus embryos and its action is reversible

We first needed to establish a concentration of cycloheximide that gives maximum inhibition of protein synthesis while also allowing subsequent survival. When animal/vegetal conjugates of the kind used for induction assays were incubated in 5, 10 or 20 μg ml^{-1} cycloheximide, protein synthesis was reduced to about 10 % of the control level within half an hour and probably sooner (Fig. 1). 1 μg ml^{-1} failed to inhibit protein synthesis to this extent, and higher concentrations than 10 μg ml^{-1} did not reduce protein synthesis below 10 %, but did cause cell death. All further work was therefore done at concentrations of 5 or 10 μg ml^{-1}. We suppose that the residual 10 % of protein synthesis which appears to be cycloheximide resistant may be mitochondrial (Jeffreys & Craig, 1976; Cascio & Wassarman, 1981).

The second condition of treatment we needed to establish was a suitable duration of exposure to the inhibitor. Conjugates incubated in cycloheximide for 6 h or more before being returned to normal medium always showed substantial cell loss. After shorter exposure times, there was considerable variation between different batches of embryos, but most showed no external signs of damage after 1 h incubation in cycloheximide, and this is the treatment we have used in subsequent experiments.

To determine the total length of time for which protein synthesis is suppressed, amino acid incorporation into acid-precipitable material was tested at intervals after conjugates had been returned to normal medium following cycloheximide treatment. As shown in Fig. 1, recovery of protein synthesis begins
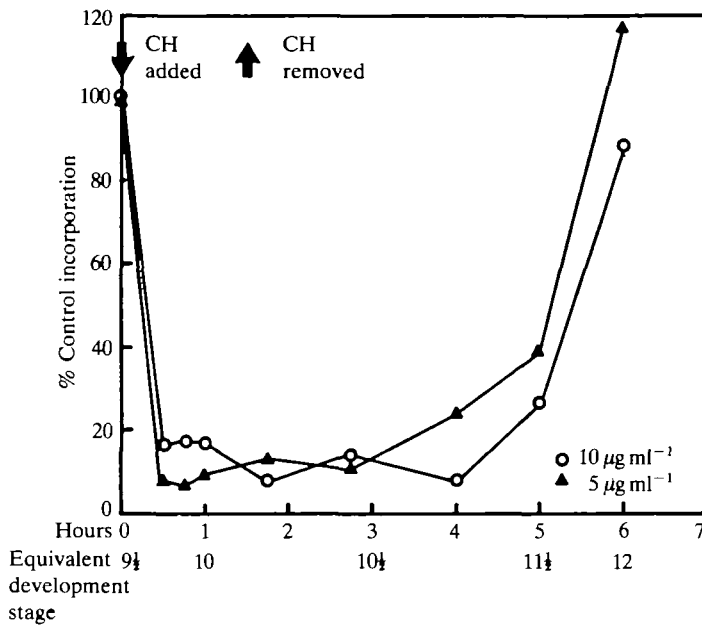
Fig. 1. Inhibition and restoration of protein synthesis by cycloheximide. Animal fragments (stage 9) and vegetal pieces (stage 8–9) were made into conjugates, allowed to attach firmly for 15 min, and then placed in medium containing cycloheximide (CH) at 5 or 10 μg ml^{-1}. After 1 h at 23 °C, they were placed in normal medium and cultured further until they reached the equivalent stages of whole embryos as shown. At various times, conjugates were incubated in [^{35}S]methionine and then frozen after h. Each value in the figure is shown at the midpoint of a h labelling period. The % control incorporation represents acid-insoluble radioactivity in cycloheximide-treated conjugates, expressed as a proportion of acid-insoluble counts in equivalent control conjugates cultured without cycloheximide. All samples contained similar amounts of total [^{35}S]methionine. The time scale has been normalized to 23 °C and the developmental stages are those of Nieuwkoop & Faber (1976).

about 3 h after removal from inhibitor, and is complete within 5 h. Therefore a 1 h exposure to cycloheximide maximally suppresses protein synthesis for about 4 h.

The third aspect of experimental design that we needed to establish was the best stage of development at which to initiate cycloheximide treatment. To use the minimum time of 7 h from conjugation to actin gene transcription, it is necessary to use animal pieces at the latest stage (Nieuwkoop & Faber (1976), stage 10) at which they are still competent to respond to vegetal induction. However, at this late stage a response is obtained only if animal and vegetal pieces remain in contact for the whole of the induction period. Since we found that vegetal pieces are more sensitive to cycloheximide than animal tissue, we prefer to employ a regime in which the inducing vegetal tissue is removed as soon as its inductive

effect has been transmitted, a process which is complete within 3 h when younger tissues are used (Gurdon, Fairman, Mohun & Brennan, 1985). We therefore decided to adopt for all future experiments a regime in which animal tissue from a stage-9 embryo is combined with stage-8 to -9 vegetal tissue; after 3 h, the vegetal tissue is removed and the remaining animal part cultured until whole embryo controls had reached stage 23. When an induction is initiated with animal tissue at stage 9 and vegetal tissue removed after 3 h, cardiac actin gene transcription is first detected 9 h after the beginning of animal/vegetal contact. Cycloheximide is added to the medium, at 5–10 μg ml^{-1}, for 1 h periods, from 0–1, 3–4 or 6–7 h after induction was initiated. It is important to note that cycloheximide treatment was equally effective in inhibiting protein synthesis at whichever of these times it was given. In every experiment we labelled a small number of embryos with [^{35}S]methionine to confirm that protein synthesis was indeed inhibited to 10 % or less of the control value. Fig. 2 summarizes the experimental design that we have followed.

The suppression of protein synthesis during induction inhibits subsequent actin gene transcription

The developmental activation of actin genes is recognized in these experiments, as in our previous work, by nuclease digestion of hybrids between messenger
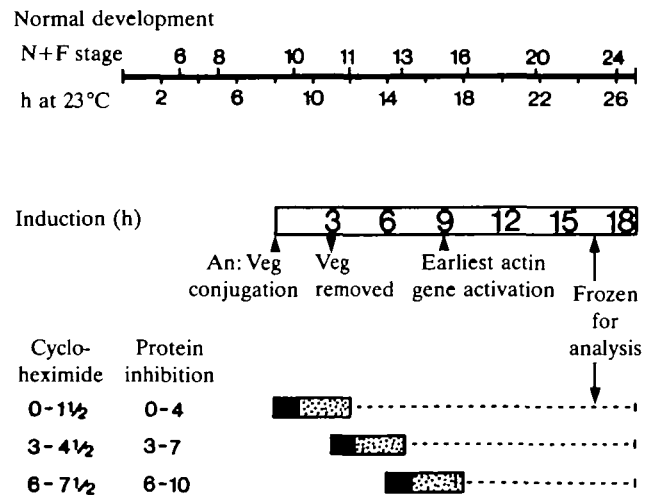

Fig. 2. Diagram of the experimental design used. Note that the 9 h induction period is commenced when animal tissue is at the late blastula stage. Incubation in cycloheximide was always for a period of 1 h; protein synthesis is maximally (i.e. 90 %) suppressed (protein inhibition) for 4 h, and fully recovered by 6 h, after the start of cycloheximide treatment. Stage numbers are those of Nieuwkoop & Faber (1976). All numbers except Nieuwkoop and Faber stages refer to hours. ■, time of exposure to cycloheximide; ▨ time after cycloheximide treatment when protein synthesis is fully inhibited.

RNA and an RNA probe. A highly radioactive anti-sense RNA probe is synthesized *in vitro* with SP6 polymerase (Melton, Krieg, Rebagliati, Maniatis, Zinn & Green, 1984), using a cDNA derived from the coding and noncoding 3′ region of cardiac actin mRNA (Gurdon *et al.* 1985). This probe has the advantage of identifying cardiac actin mRNA which is formed in muscle as a result of induction, as well as cytoskeletal actin mRNA, which is synthesized in all cells at about the same stage in development by a noninductive process (Gurdon *et al.* 1985).

Fig. 3 illustrates the results of an experiment carried out according to the design seen in Fig. 2. We chose to analyse material when it had reached the equivalent of control stage 23; this is considerably later than the time of initial actin gene transcription at stage 11–12, but has the advantage of providing a strong signal from single embryo fragments, as can be seen by comparing stage-9 and stage-23 whole embryo analyses in Fig. 3C,D. Induced animal fragments, cultured in the absence of cycloheximide, show as strong activation of cardiac actin genes as whole embryos (Fig. 3E,F). Cardiac actin gene transcription is totally suppressed by cycloheximide treatment from 0–1h or 3–4h (Fig. 3G–L), equivalent to protein synthesis inhibition from 0–4 or 3–7h. However, cardiac actin gene transcription is partly and variably, but by no means fully, suppressed by treatment from 6–7h, when protein synthesis is inhibited from 6–10h just before actin transcription starts (Fig. 3M–O). We conclude that the transcriptional activation of the cardiac actin gene by induction absolutely requires protein synthesis during the first two thirds of a 9h induction period, but becomes
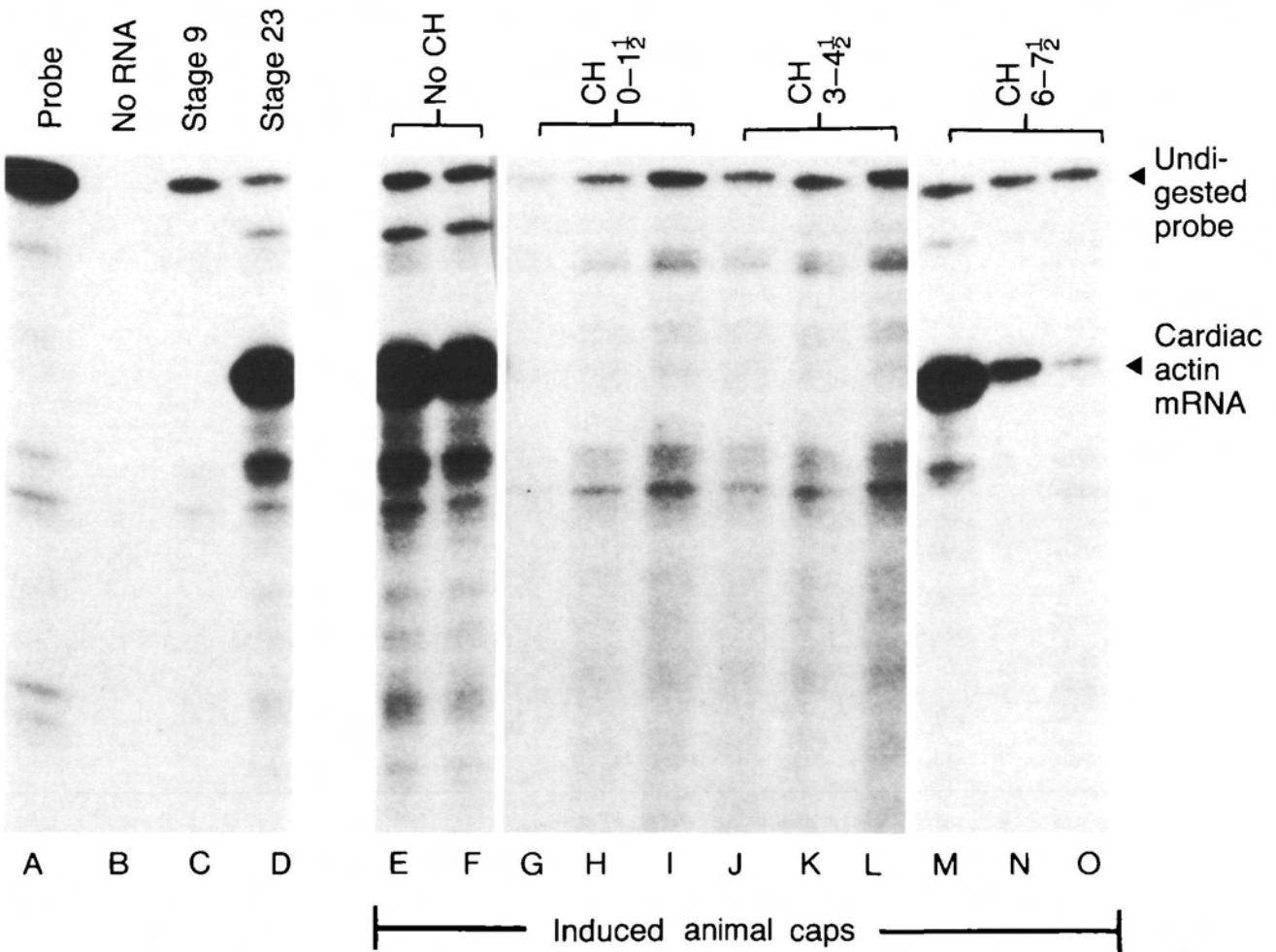

Fig. 3. Transcript analysis of cycloheximide effects on cardiac actin gene activation by induction. The anti-sense ^{32}P-RNA probe used in these experiments is complementary to part of the 3′ untranslated and coding regions of cardiac actin mRNA, and has been described by Gurdon *et al.* (1985). The unprotected probe is 380 nucleotides long; 285 nucleotides of this is protected from RNase digestion by cardiac actin mRNA. Tracks A–D show the probe and analyses of whole embryo RNA at stage 9 and 23. Tracks E to O show analyses of RNA from induced animal caps which have been treated with no cycloheximide or with cycloheximide (CH) for the periods of time indicated, as explained diagrammatically in Fig. 2. Each of the tracks E to O represents the analysis of RNA from a single animal cap.

independent of it at about the time when transcription starts. We assume that protein synthesis is also required for all other muscle-specific genes activated by mesodermal induction.

This result raises the question whether the protein synthesis requirement we have identified applies only to genes activated by induction or to all genes whose transcription is initiated at this stage of development. This can be answered by the same design of experiment carried out with a cytoskeletal actin gene. As mentioned above, the same SP6-generated anti-sense RNA probe as is used to identify cardiac actin transcripts is protected from RNase digestion for a shorter length by cytoskeletal actin mRNA. There is a small amount of maternally synthesized cytoskeletal actin mRNA present in eggs and early embryos, but the amount of this increases about tenfold between stages 9 and 23 when new transcription of these genes starts during gastrulation (Cascio & Gurdon, 1986). The same increase is observed in isolated animal caps, in which no induction takes place, as in animal/vegetal conjugates (compare Fig. 4A, tracks 1–3 and 7–9 with Fig. 4B, tracks 1–3 and 7–9). The transcriptional activation of these genes is therefore independent of induction. This developmental increase is totally inhibited by a 0–1 h cycloheximide treatment (or 0–4 h protein synthesis inhibition) starting at stage 9, in induced as well as in uninduced animal caps (Fig. 4A, tracks 4–6, and 4B, tracks 4–6). We conclude that protein synthesis is required for the initial activation of actin gene transcription, whether by an inductive or noninductive process. Indeed, as we see in the next section, protein synthesis at the late blastula and early gastrula stages seems to be required for the activation of all genes whose transcription first starts during gastrulation.

The inhibition of protein synthesis at the late blastula stage has a highly selective effect on subsequent gene expression

A major concern in interpreting inhibitor experiments is whether the effect is specific or not. If the cycloheximide treatment we have used is having an unspecifically toxic effect, we would expect the synthesis of many different proteins to be partially or wholly inhibited, irrespective of whether they depend on new transcription or not. If, conversely, cycloheximide is specifically inhibiting subsequent transcription, we would expect only newly transcribed genes (genes never before transcribed) to have their expression inhibited, once recovery from cycloheximide has taken place. We have distinguished these two interpretations by 2D analyses of newly synthesized proteins.

Fig. 5 shows 2D gel analyses of proteins labelled with [35S]methionine for 4–6 h when control embryos
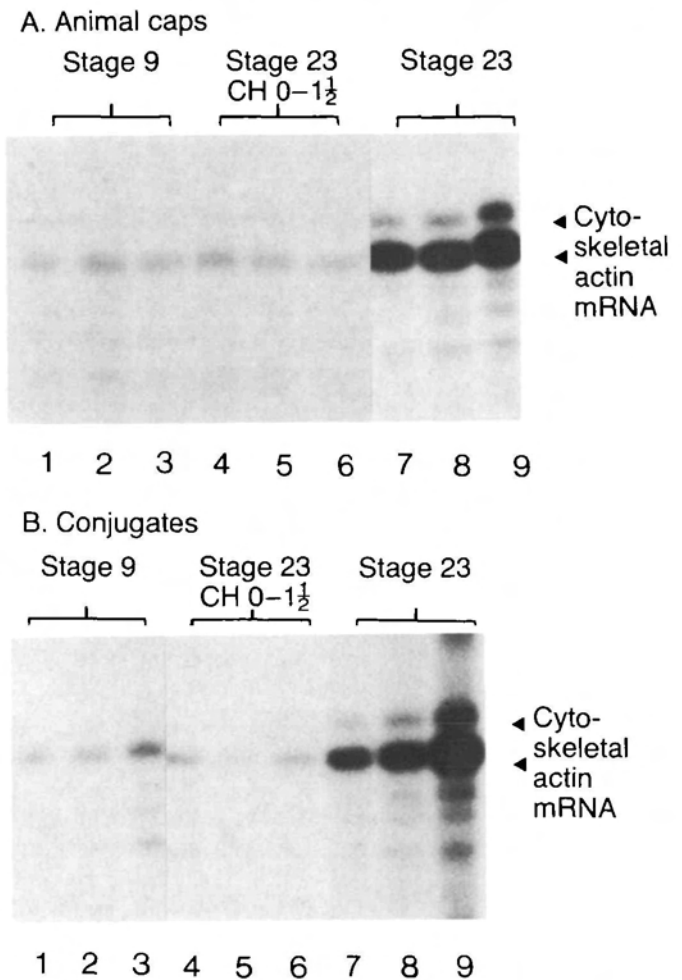

Fig. 4. Transcript analysis of cycloheximide effects on cytoskeletal actin gene activation. The same 32P-RNA probe was used as is described in Fig. 3; about 135 nucleotides of this 380-nucleotide probe is protected by cytoskeletal actin mRNA (Gurdon *et al.* 1985). In both uninduced animal caps (A) and in induced animal caps or conjugates (B), a low abundance of maternal transcripts (tracks 1–3) is increased by about tenfold by stage 23 (tracks 7–9). This increase is totally prevented by a 1 h exposure to cycloheximide at stage 9 (tracks 4–6). All of the tracks shown are analyses of RNA from a single animal cap or conjugate.

are undergoing gastrulation and neurulation. The range of proteins synthesized by induced animal caps is very similar, whether or not they were incubated in cycloheximide for 0–1 h of the induction period (compare Fig. 5B, no cycloheximide, with Fig. 5C, cycloheximide treated). In accordance with the RNA analyses described in the previous section, the cytoskeletal actin spot is present in both samples, but is weaker in the cycloheximide case (Fig. 5C) than in the control (Fig. 5B), the former presumably reflecting the translation of maternal transcripts as seen in Fig. 5A. Similarly, the muscle-specific α-actin spot, largely composed at this stage of development of
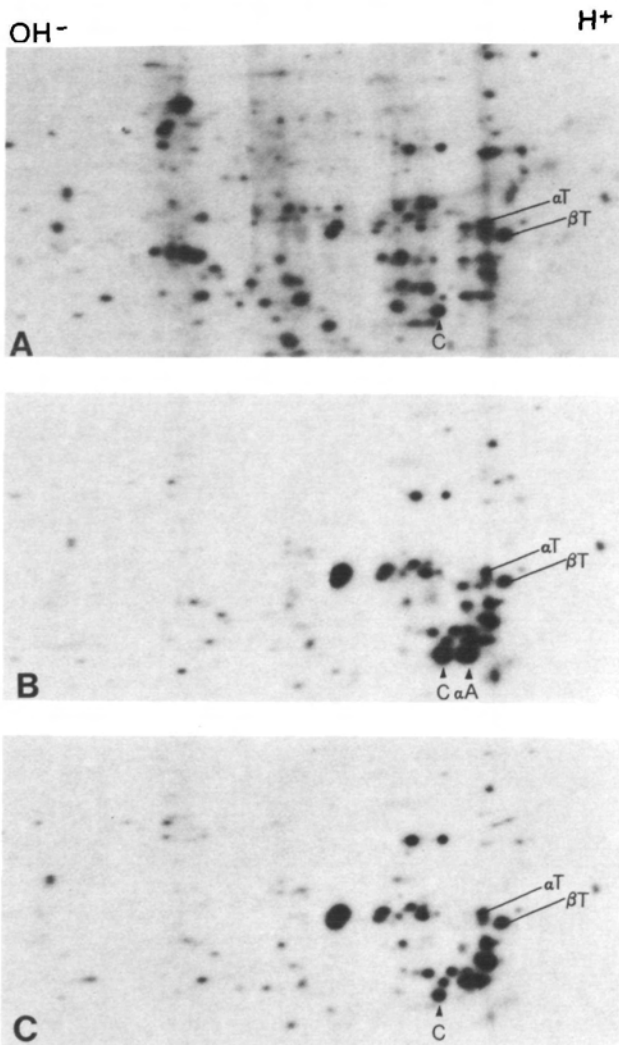
OH⁻   H⁺

Fig. 5. Two-dimensional gel analysis of proteins synthesized after cycloheximide treatment.
(A) Uninduced animal caps labelled with [^{35}S]methionine for 4 h from stage 9; no cycloheximide treatment.
(B,C) Induced animal caps, labelled with [^{35}S]methionine for 6 h from the equivalent of stages 17 to 23. (B) No cycloheximide treatment. (C) Cycloheximide from 0–1 h after conjugation. αT, βT, spots provisionally identified as α and β tubulin (Brock & Reeves, 1978; Mohun, 1980). C, cytoskeletal actin; αA, α actins, including cardiac and skeletal actins. Each figure represents the analysis of three or fewer animal caps, containing $0.5–1.0\times10^6$ cts min^{-1} of labelled protein.

cardiac and skeletal actins, is absent from the cycloheximide sample. The very faint spot in the α-actin position in Fig. 5C is almost certainly not α-actin, for two reasons: (1) the same spot is seen in some blastula analyses (Fig. 5A) when muscle actins are not synthesized and (2) SP6 transcript analyses on equivalent samples show no cardiac actin mRNA (previous section). The great majority of other spots appear the same in Fig. 5B and C.

To make this assessment more quantitative, we have scored all spots that can be detected in one-week autoradiographs of the 2D gels. We have been able to compare some 200 spots in three kinds of samples, which include uninduced animal caps labelled at the blastula stage (Fig. 5A), as well as induced animal caps, with or without cycloheximide, labelled at the equivalent of a late neurula stage. The results of this analysis are summarized in Table 1, from which the following conclusions can be drawn. When an induced animal cap at control stage 23 is compared to a stage-9 animal cap (uninduced), just under 10 % of the proteins recognized are unique to each sample. This means that about 10 % of the proteins synthesized at the late blastula stage have ceased to be synthesized by stage 23, and that only 10 % of the proteins synthesized by a stage-23 conjugate were not already being synthesized at the blastula stage. The second comparison is between a normal conjugate and a cycloheximide-treated conjugate, both at stage 23. The second row of Table 1 permits two conclusions. One is that cycloheximide does not induce the synthesis of any new proteins. The other is that cycloheximide suppresses about 10 % of the proteins synthesized by an uninhibited stage-23 conjugate. The third row of Table 1 yields another conclusion, namely that a cycloheximide-treated conjugate synthesizes hardly any proteins that were not already being synthesized in a blastula. This in turn tells us that cycloheximide treatment at the late blastula stage prevents the synthesis of nearly all new proteins that would normally appear up to stage 23. A detailed comparison of individual spots confirms this point.

Our 2D gel analysis of newly synthesized proteins in these experiments is consistent with previous analyses of others, e.g. Brock & Reeves (1978), Ballantine, Woodland & Sturgess (1979), Bravo & Knowland (1979), Sturgess, Ballantine, Woodland, Mohun, Lane & Dimitriadis (1980), Smith & Knowland (1984) and Smith (1986) on normally developing *Xenopus* embryos. However, our results cannot be compared exactly with previous work, partly, because so many spots on 2D gels have not before been comprehensively scored and, partly, because we have intentionally classified all spots only as present or absent. Thus spots that are a few times weaker in one of the two gels being compared would be recorded by us as present. As a result, our analysis concentrates on proteins whose synthesis is strengthened or weakened at least five times. We assume that the 10 % of new proteins that start to be synthesized between stages 9 and 23 are products of genes that are newly transcribed at some time after the mid-blastula transition, rather than of maternal messages newly translated after stage 9.

Table 1. *Inhibition of protein synthesis during early gastrulation prevents the transcriptional activation of developmentally regulated genes: summary of two-dimensional protein gel comparisons*

	Numbers of two-dimensional gel spots		
Comparison	Shared	Unique to	Unique to
st. 21 conjugate and st. 9 animal cap	226	st. 21 conj. 20 (8·8%)	st. 9 an cap 20 (8·8%)
st. 21 conjugate and st. 21 conjugate + CH	234	st. 21 conj. 18 (7·7%)	st. 21 conj. + CH 0 (0%)
st. 21 conjugate + CH and st. 9 animal cap	198	st. 21 conj. + CH 4 (2%)	st. 9 an cap 15 (7·6%)

Animal tissues or conjugates were injected with [^{35}S]methionine at 10 mCi ml^{-1}, incubated for 3 h from stage 9–10 or for 6 h from stage 17–23 (average stage 21) and the extracted proteins resolved on 2D gels. Some conjugates were incubated for 1 h in cycloheximide-containing medium (CH) as soon as the conjugate was made at about stage 9. Some 200 or more spots were matched in samples being compared. The last two columns show the numbers (and %) of spots unique to each member of the pair being compared.

The main conclusion from 2D gel analysis is that cycloheximide treatment irreversibly inhibits only a small proportion of proteins synthesized in early development. Nearly all of the proteins synthesized by a postneurula embryo were rapidly inhibited by cycloheximide, but 90% of these, in fact all those already being synthesized before cycloheximide treatment, soon recover to normal levels of synthesis. We therefore regard the cycloheximide effect as highly selective on newly transcribed genes and not a consequence of general toxicity.

The immediate effect of cycloheximide is not on DNA synthesis or RNA synthesis

Cycloheximide is one of the most widely used inhibitors of protein synthesis and is particularly valuable on account of the ready reversibility of its effect. It usually acts by arresting the movement of ribosomes along messenger RNA (Vasquez, 1979). As soon as the inhibitor is removed, ribosomes recommence their movement along, and translation of, previously existing messenger RNAs. It is remarkably nontoxic, insofar as protein synthesis can be arrested for at least 4 h in cultured amphibian cells and yet these cells will fully recover and continue growth and division when the inhibitor is removed (J. R. Tata, personal communication).

It might be argued that the effect of cycloheximide in our induction experiments is indirect. For example cycloheximide might inhibit the synthesis of proteins needed for replication, thereby preventing cell division or DNA synthesis; this in turn might prevent new gene transcription if quantal cell division or DNA synthesis is required (Dienstman & Holtzer, 1975). We have demonstrated before that cell division is not required for the induced transcription of muscle actin genes (Gurdon & Fairman, 1986). Table 2 shows that cycloheximide reduces DNA synthesis to

only about one third of the control level, when in the same experiment it reduced protein synthesis by nearly 20 times. Therefore the inhibitory effect of cycloheximide cannot be attributed solely to an indirect effect of reduced DNA synthesis.

Can the cycloheximide effect be explained as an indirect consequence of inhibited RNA synthesis? It could be argued that cycloheximide treatment at the late blastula stage inhibits all further transcription and hence the synthesis of all new proteins, by removing the supply of some universal transcriptional component. This idea has been eliminated by labelling embryo fragments after recovery from cycloheximide inhibition and finding no significant reduction in RNA synthesis compared to uninhibited controls (results not shown). However, 95% of the labelled

Table 2. *Cycloheximide reduces but does not eliminate DNA synthesis in* Xenopus *embryo fragments*

^{3}H-TdR labelling (duration in h from stage 9)	Controls % acid insol.	Cyclohex. (0–1·5): % acid insol.	DNA synthesis: cyclohex. as % of controls
0–2	33	13	38
2–4	26	7	25
4–6	31	12	37
6–8	30	16	51

Conjugates consisting of stage 9 animal caps and stage-8 to -9 vegetal pieces were incubated in [^{3}H]thymidine-containing medium (0·83 mCi ml^{-1}) for the time shown in the left-hand column. In 2 h each sample of two animal fragments took up a few hundred thousand counts, of which the percentage shown became acid insoluble by incorporation into DNA. Cycloheximide treatment for the first 1 h of culture reduced DNA synthesis to about one third of controls (last column), but in the same experiment equivalent embryo fragments had their incorporation of labelled amino acid into protein reduced by cycloheximide to an average of 6% of the controls (not shown).

Table 3. *Poly(A)⁺ RNA synthesis in embryo fragments that have recovered after cycloheximide treatment*

Labelled RNA	Cycloheximide-treated	Untreated controls
poly(A)⁻	13 130 dpm	12 320 dpm
poly(A)⁺	987 dpm	460 dpm
poly(A)⁺ as % of total	6·9 %	3·6 %

Animal/vegetal conjugates were made at stage 9, incubated in 5 μg ml⁻¹ cycloheximide for 1 h and cultured in normal medium until control stage 16. They were then injected with [³H]uridine, which was also added to the medium. After a further 8 h, at control stage 24, they were frozen for analysis and their RNA fractionated by a poly U sepharose column. The results show the analysis of 15 cycloheximide-treated conjugates and 15 controls. It is doubtful whether the higher than average value for poly(A)⁺ RNA in the cycloheximide-treated sample is significant. dpm, disintegrations min⁻¹.

RNA in such an experiment is ribosomal RNA and tRNA, the products of transcription by polymerases I and III. This does not eliminate the possibility that all polymerase II activity, required for mRNA synthesis, might be irreversibly inhibited by cycloheximide. There are two indirect arguments against this proposition. One is that embryos or embryo fragments completely dissociate at the mid-gastrula stage (which they did not do in our experiments) if RNA polymerase II activity is eliminated by α-amanitin (Lee, Hynes & Kirschner, 1984). The other is that cycloheximide treatment from 6–7 h in our experiments variably reduced, but never eliminated, actin gene transcription, even though it did inhibit protein synthesis to the maximal extent. We have also investigated this question directly, by measuring the synthesis of poly(A)⁺ RNA in animal/vegetal conjugates, which had recovered from cycloheximide treatment, and in equivalent untreated controls. The values in Table 3 show that embryo fragments treated with cycloheximide during stage 9 had regained a normal level of poly(A)⁺ RNA synthesis.

We conclude that cycloheximide is likely to have inhibited protein synthesis in our experiments directly and by its most widely recognized mode of action. There was no permanent or irreversible inhibition of transcription by polymerase II. Our results cannot therefore be explained by indirect effects on DNA or RNA synthesis.

Discussion

The main result of the experiments we have described is that cycloheximide eliminates the expression of just those genes whose transcription is normally initiated

a few hours after the time of cycloheximide treatment. How far can we interpret this inhibitory effect?

Our observations show that, after cycloheximide is removed, embryo fragments survive well, that protein synthesis recovers to its normal level and that 90 % of the kinds of proteins made before inhibition are again made after it. This means that the brief treatment we have used does not have a generally toxic effect. Similarly the selectivity of its action, inhibiting only those proteins whose synthesis needs new transcription, argues against unspecific effects. We assume that cycloheximide is working in our experiments by its direct effect on protein synthesis. We have seen that protein synthesis is shut down within 30 min of treatment and probably sooner, whereas DNA synthesis is reduced to a much smaller extent. The fact that 10 % of protein synthesis is resistant to cycloheximide, as is commonly observed (Cascio & Wassarman, 1981), does not affect our conclusions, since these depend on inhibition not resistance.

What further conclusions can be drawn from our results? The least interesting interpretation is that the inhibition of new gene expression after cycloheximide treatment results from an irreversible block to further transcription. If this had occurred, the observed synthesis of all those proteins that were previously being synthesized at the late blastula stage could be explained by the translation of persisting mRNA. However, we have demonstrated the restoration of a normal rate of total, as well as poly(A)⁺, RNA synthesis. Therefore the cycloheximide effect appears to be quite specific, affecting only those genes whose expression had not yet been initiated at the time of protein synthesis inhibition.

Those components whose synthesis is inhibited by cycloheximide appear to be needed not only for the transcription of genes activated by induction, such as muscle-specific actin, but also for ones activated by other means, such as cytoskeletal actins. This reduces the likelihood that the inhibited components are cell surface or other molecules needed for cell–cell interactions. The most interesting, but by no means only, possibility is that the proteins are transcription factors. Naturally it would be of great interest to identify the genes concerned and their products. One route that could achieve this is a careful search through a cDNA library, constructed from messenger RNA of early gastrula embryos, using the kind of approach described by Sargent & Dawid (1983).

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