Volume 16 Number 11 1988 Nucleic Acids Research

Trans-activation of transcription, from promoters containing immunoglobulin gene octamer sequences, by myeloma cell mRNA in *Xenopus* oocytes

Glen E.Sweeney and Robert W.Old

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

Received February 17, 1988; Revised and Accepted April 11, 1988

ABSTRACT

To study factors required for immunoglobulin gene transcription hybrid promoters were made by linking octamer elements to a Xenopus albumin gene construct containing only 50bp of the albumin gene promoter. When injected into oocytes these hybrid promoters directed transcription far less efficiently than the unmodified 50bp albumin gene promoter fragment. Activity of the hybrid promoters, but not the unmodified albumin promoter, could be stimulated by preinjection of poly(A)$^+$ RNA from NS1 myeloma cells. This stimulation may be caused by translation of the NS1 poly(A)$^+$ RNA into transcription factors that act on the octamer. Both the reduction in transcription caused by octamer insertion and the extent of the inducibility by NS1 RNA are greater when two, rather than one, octamers are inserted.

INTRODUCTION

The mammalian immunoglobulin heavy chain variable region (V_H) genes are specifically expressed in β-lymphocytes. This expression is dependant on both a cell-type-specific enhancer, located in the large intron, and a cell-type-specfic promoter (1,2,3,4,5). The octamer sequence ATTTGCAT, or its inverse complement, is found upstream of all human and mouse immunoglobulin genes and is also present in the mouse heavy chain enhancer (6). In addition it is found upstream of some genes expressed in a wide variety of tissues such as the human histone H2B gene and the human U1 and U2 small nuclear RNA genes (7,8).

Deletion analysis and mutagenesis have shown that the octamer is essential for the cell-type-specificity of the V_H gene promoter (4,5,9). Furthermore if a synthetic octamer is linked to a heterologous promoter that includes a TATA box then a B-cell-specific promoter is produced (10,11). Mutations at any base in the octamer sequence of such constructs reduce or abolish promoter activity in B-cells (11).

Gel-retardation experiments have identified at least two factors that bind to the octamer in vitro. One of these, sometimes referred to as NFA1 or

OTF1 appears to be fairly ubiquitous (12,13), whilst the other, sometimes known as NFA2 or OTF2 is only found in B-cells (14,15). It is difficult to correlate these *in vitro* binding assay with *in vivo* function, although there is evidence from *in vitro* transcription experiments using nuclear extracts that NFA1/OTF1 may be required for activity of the human histone H2B promoter whereas the B-cell-specific factor NFA2/OTF2 may be needed for expression of immunoglobulin genes (13,15).

The *Xenopus* oocyte is an attractive system in which to assay putative transcription factors. In such assays oocytes are coinjected with a particular gene and with extracts made from nuclei in which the gene is transcribed. If the extracts contain specific transcription factors then expression of the gene in coinjected oocytes will be stimulated relative to expression in oocytes injected with the gene alone. This approach has been used to partially purify factors needed for transcription of sea urchin histone H2B genes (16,17). It has also been found that the expression in oocytes of the erythroid-specific histone H5 gene from chicken can be stimulated by coinjection of nuclear extracts from chicken erythroid cells (18). A refinement of this technique involves injecting oocyte cytoplasm with mRNA prepared from cells in which the gene under investigation is expressed. The oocytes are incubated for a period to allow the injected RNA to be translated and then the gene under investigation is injected into the oocyte nucleus. If the mRNA is translated to give factors which are normally absent in oocytes, but which are required for maximal transcription of the gene, then transcription will be more efficient in oocytes preinjected with mRNA than in oocytes not preinjected. Hence the oocyte is being used to assay for mRNAs that encode transcription factors. The feasibility of this approach has been demonstrated by Maxson *et al.* (17), who found that transcription of the sea urchin late histone H2B gene in oocytes could be stimulated 12-fold by prior injection with sea urchin mRNA.

Assaying for mRNAs that encode transcription factors, rather than assaying the factors themselves, has the advantage that it is possible to use the assay to identify cDNA clones of transcription factors by transcribing batches of the clones *in vitro* and testing the transcripts by the oocyte assay. Such an approach has been used to isolate cDNA clones of an mRNA whose injection has a direct effect on oocyte electro-physiology, namely the mRNA encoding bovine substance-k receptor (19).

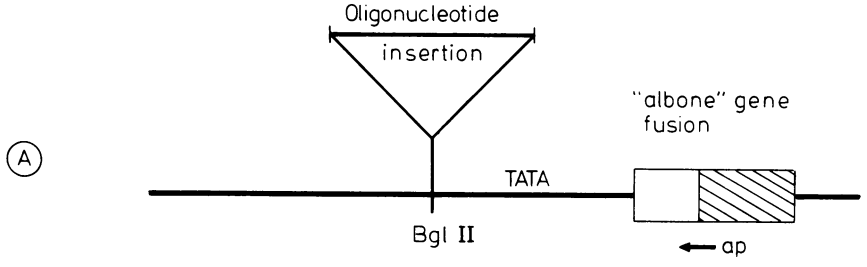
As a first step towards cloning factors required for the B-cell-specific

expression of immunoglobulin genes we show that injection of myeloma cell mRNA stimulates activity of the Xenopus 68kdal albumin gene promoter which has been modified by the addition of one or two octamer motifs. Although the addition of octamer motifs renders the promoter inducible by factors encoded by (or present in) myeloma cell mRNA, it also causes a very substantial decline in the efficiency of the promoter. Both the inducibility of the modified promoter and the reduction in efficiency compared to the unmodified promoter, are greater when two tandem octamer sequences are inserted rather than a single octamer.

MATERIAL AND METHODS.

Construction of octamer clones.

The parent clone used in this study was the albumin promoter construct Δ-50. This is an M13 clone containing sequences of the Xenopus 68kdal albumin gene (20) (from -50 nt from the transcriptional start site to 50 nt within the first exon) fused with a 3' terminal region of a Xenopus histone H3 gene (a 0.37 kb Bam HI fragment containing the 3' terminal 5



B

Octamer

OCT 1:—— A G A T C T T A T T T G C A T G A T C T

Octamer

OCT 2:—— A G A T C T T A T T T G C A T G A T C T T A T T T G C A T G A T C T

Figure 1. Structure of the mIgOCT clones. a) oligonucleotides containing an octamer element were inserted into the BglII site of albumin Xenopus 68kdal gene promoter construct Δ-50. The Bgl II site is the position of the fusion between albumin DNA and vector DNA. The open box represents DNA from the first exon of the albumin gene, the hatched box represents DNA from the Xenopus histone H3 gene, ap denotes the site of binding of the albone primer. b) the sequences of the octamer inserts in mIgOCT1 and mIgOCT2.

codons and a relatively large 3' flanking sequence (21)). The albumin promoter sequences in this construct therefore extend from the BglII site at about -50, to within the albumin gene's first exon where the junction with the histone H3 gene sequence has been made (fig.1a). In order to insert octamer elements two oligonucleotides were synthesised with the sequences GATCATGCAAATAA and GATCTTATTTGCAT. These were then phosphorylated with ATP and polynucleotide kinase and annealled to each other by heating at 65°C for 10 minutes and allowing to cool to room temperature. This produced a 10bp piece of double stranded DNA, including an octamer element, that was flanked by sticky ends compatible with those produced by Bgl II digestion. This fragment was then ligated into Δ-50 RF that had been cleaved with Bgl II. Two of the resulting recombinants were selected for use in oocyte injection experiments: mIgOCT1, which has a single octamer insert, and mIgOCT2, which has two octamer elements inserted in tandem (fig.1b).

Preparation of mRNA from NS1 Myeloma cells.

NS1 cells (22) were grown in RMPI 1640. To prepare RNA, cells were washed twice in PBS and then resuspended in 10ml of lysis buffer (0.14M NaCl, 1.5mM $MgCl_2$, 10mM tris-Cl [pH8.6], 0.25% Nonidet P40) per 500 ml of cell culture. The lysate was centrifuged for 1 minute at 3,000 rpm and the pelleted nuclei were discarded. An equal volume of 2 x proteinase k buffer (0.2M tris-Cl[pH7.5], 25mM EDTA, 0.3M NaCl, 2% SDS) was combined with the supertnatant and 0.1 volumes of proteinase k solution (2mg/ml) were added. The mixture was incubated for 30 minutes at 37°C and extracted twice with an equal volume of phenol/chloroform. RNA was then precipitated with ethanol. $Poly(A)^+$ RNA was prepared by oligo-dT-cellulose chromatography (23).

Preparation of mRNA from Xenopus liver. Liver RNA was prepared from the livers of adult male frogs by the guanidinium isothiocyanate/caesium chloride method (24). $Poly(A)^+$ RNA was prepared by oligo-dT-cellulose chromatography (23).

Oocyte Injections.

For RNA injections 20 nl of 0.5 mg/ml poly $(A)^+$ RNA solutions were injected into the oocyte cytoplasm. For DNA in injections 20 nl of 0.5 mg/ml solutions of RF DNA were injected into oocyte nuclei. Oocytes were incubated for 24 hours at 20°C in Barth-X (25) following DNA injections.

Preparation of nucleic acids from injected oocytes.

To extract nucleic acids, up to 20 oocytes were homogenised in 0.5 ml of

NAE (0.3M sodium acetate pH6.5, 1mM EDTA) and 50 μl of a 20% solution of SDS was added. The homogenate was then extracted with equal volumes of phenol and phenol-chloroform and nucleic acids were precipitated with ethanol.

Primer extensions.

Transcripts from albone Δ-50 and its derivatives were detected using a 17 mer primer complementary to nucleotides spanning the junction between albumin and histone sequences. The sequence of this primer is CGCCTCGGATCCACCTTC. Primers were end-labelled with γ^{32}P-ATP and polynucleotide kinase, separated from unincorporated label on Sephadex G50 columns, precipitated with ethanol and resuspended in 3x primer extension hydridisation buffer (1x buffer is 0.4M NaCl, 10mM PIPES [pH6.4], 0.5mM EDTA). For extension reactions, 100pg of end-labelled primer was annealled to 20μg of RNA in 1x primer extension hybridisation buffer by overnight incubation at 45°C. Nucleic acids were then recovered by ethanol precipitation and resuspended in 20μl of primer extension reaction buffer (50mM Tris-Cl [pH8.3], 6mM $MgCl_2$, 10mM DTT, 1mM each dNTP). Annealled primers were extended by the addition of 10 units of reverse transcriptase followed by incubation at 37°C for 40 minutes. Extension products were fractionated by electrophoresis through urea /8% acrylamide gels, and detected by autoradiography.

Southern Transfers.

Nucleic acid from injected oocytes was electrophoresed in an agarose gel and transferred to nylon membrane as described (26). The transfer was hybridised with a radiolabelled M13 mp18 probe.

RESULTS

Expression of mIgOCT clones in oocytes with and without preinjection of NS1 cell mRNA.

mIgOCT1 and mIgOCT2 are albumin promoter constructs modified by the insertion of octomer elements. The promoter region in these two constructs consists only of one or two octamer sequences (mIgOCT1 and mIgOCT2 respectively) located directly upstream of the first 50bp of the Xenopus 68kdal albumin gene (fig. 1). Similar promoters have been shown to be lymphoid-specific (10,11), with this specificity being entirely due to the presence of the octamer. Hence it might be expected that expression of the mIgOCT clones will be stimulated by factors encoded by NS1 cell mRNA. To find out whether or not this is the case, experiments were carried out in which oocytes were given cytoplasmic injections of NS1

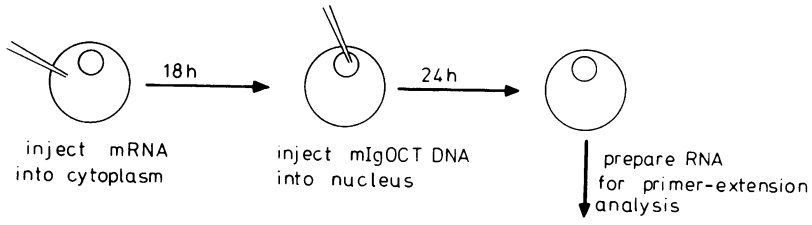


Figure 2. Assay for mRNAs encoding transcription factors required for mIgOCT expression. 10ng of NS1 poly(A)$^+$ RNA were injected into oocyte cytoplasm, after which oocytes were incubated at 20^{o}C for 18 hours. 10ng of mIgOCT DNA was then injected into oocyte nuclei. After a 24 hour incubation at 20^{o}C RNA was prepared from the oocytes and mIgOCT transcripts were assayed by primer extension using the albone primer.

cell mRNA, and incubated for 18 hours. The nuclei of these oocytes, and of uninjected control occytes were then injected with mIgOCT DNA and transcription of the DNA was monitored by primer extension analysis using the albone primer (fig. 2). The results of an experiment of this kind are shown in figure 3. Lanes 1 and 2 each show the primer extension products given by 20μg RNA (equivalent to the content of 4 oocytes) from a batch of 20 oocytes each given nuclear injections of 10ng RF DNA from the parent clone of the mIgOCT constructs, Δ-50. The oocytes used in lane 1 have been given an earlier cytoplasmic injection of 20 ng NS1 cell poly(A)$^+$ RNA and incubated at 20^{o}C for 18 hours. The oocytes used in lane 2 had been similarly incubated, but had not received any RNA. It can be seen that the DNA is efficiently transcribed in both sets of oocytes giving large amounts of correctly initiated RNA (arrowed) as well as many incorrectly initiated transcripts. Preinjection of the oocytes with NS1 poly(A)$^+$ RNA does not affect either the accuracy or the efficiency of transcription.

The results of primer injection analysis of RNA from oocytes similarly injected with mIgOCT1 DNA can be seen in lanes 3 (oocytes preinjected with NS1 poly(A)$^+$ RNA) and 4 (oocytes not preinjected). Obviously the amount of RNA produced by injection of this DNA is much lower than is produced by injection of Δ-50 (although the accuracy of transcription is superior). Furthermore the amount of RNA produced is greater in oocytes preinjected with NS1 poly(A)$^+$ RNA than in oocytes not preinjected.

The results of primer extension analysis of RNA from oocytes injected with mIgOCT2 DNA can be seen in lanes 5 (oocytes preinjected with Xenopus liver poly(A)$^+$ RNA), 6 (oocytes preinjected with NS1 poly(A)$^+$ RNA) and 7

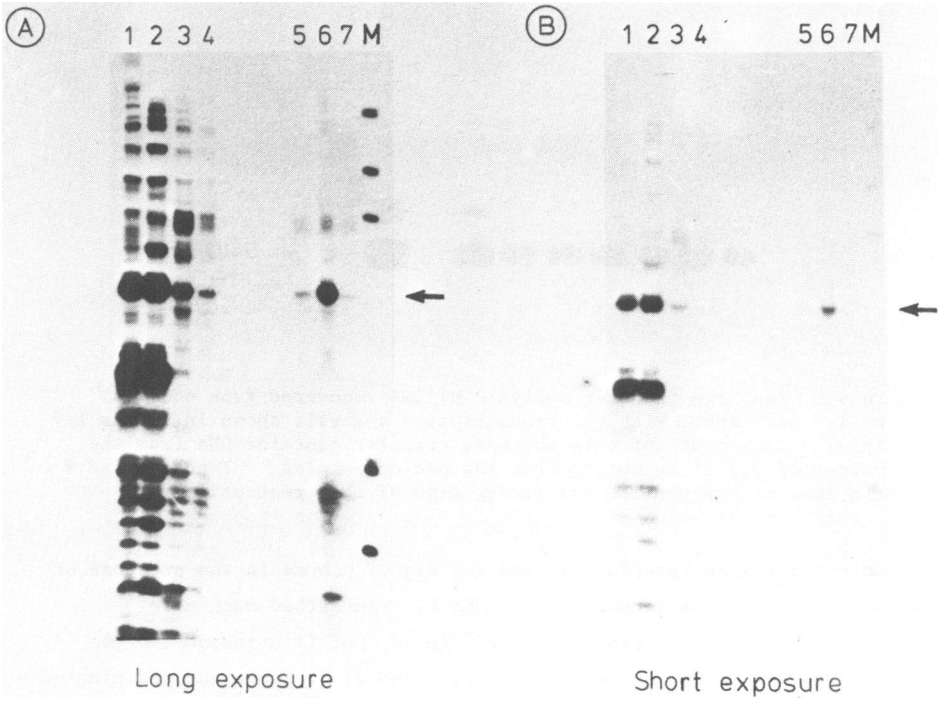


Figure 3. Activation of mIgOCT promoters by preinjection of NS1 cell mRNA. To allow a fuller appreciation of the data 24 hour (a) and 4-day (b) exposures of the gel are shown. End-labelled albone primer was annealed to 20μgsamples of RNA from oocytes whose nuclei had been injected with 10ng RF DNA from the parent clone Δ-50 (lanes 1 and 2), mIgOCT1 (lanes 3 and 4) and mIgOCT2 (lanes 5,6 and 7). All the oocytes were incubated for 18 hours at 20°C prior to nuclear injection. At the start of this period oocytes used to make the RNA analysed in lanes 1,3 and 6 were given cytoplasmic injections with 10ng of NS1 poly(A)$^+$ RNA, and the oocytes used to make the RNA analysed in lane 5 were similarly preinjected with 10ng Xenopus liver poly(A)$^+$ RNA. Extension products were fractionated through 8% sequencing gels and located by autoradiography. Markers (M) were end-labelled fragments from a digest of pBR322 with HpaII. The arrow indicates correctly initiated transcripts.

(oocytes not preinjected). It can be seen that there is very little transcription of mIgOCT2 in oocytes unless they have received a prior injection of NS1 poly(A)$^+$ RNA. Hence both mIgOCT1 and mIgOCT2 are transcribed less efficiently than their parent clone Δ-50. In addition activity of both constructs is higher in oocytes given a prior injection of NS1 RNA than in oocytes not so injected. The magnitude of these two effects is clearly greater when mIgOCT2 DNA is injected than when mIgOCT1 DNA is injected.

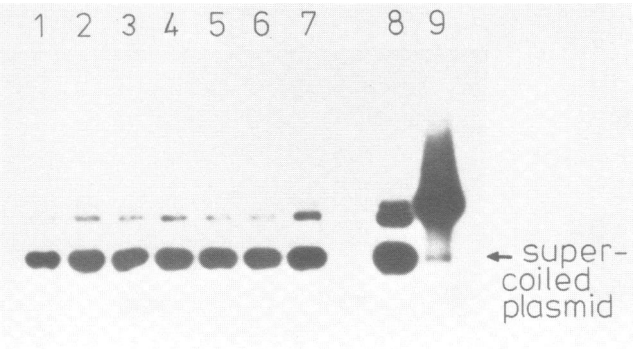


Figure 4. Southern transfer analysis of DNA recovered from oocytes. Tracks 1-7 correspond with the transcription analysis shown in tracks 1-7 of fig.3. Each track of this southern transfer contains DNA from the equivalent of 0.3 of an oocyte from the pooled samples. Tracks 8 and 9 contain 10ng of supercoiled and linear Δ-50 RF DNA, respectively.

The only difference between Δ-50 and the mIgOCT clones is the presence of octamer motifs in the latter. Δ-50 DNA is transcribed much more efficiently in oocytes than the mIgOCT clones, but is unresponsive to prior injection of NS1 RNA (fig.3, lanes 1 and 2). Hence the addition of octamer sequences to the albumin promoter fragment present in Δ-50 causes a marked reduction in the efficiency of the promoter, but also renders it inducible to factors encoded by (or present in) NS1 poly(A)$^+$ RNA. These effects are more marked when two octamer motifs are present as in mIgOCT2. It should be noted that prior injection of Xenpous liver poly(A)$^+$ RNA does not lead to increased expression of mIgOCT2 DNA (fig3 lane 5). This shows that the stimulatory effect of injection of NS1 poly(A)$^+$ RNA is not simply an artefact caused by the physical effect of cytoplasmic injection. It also shows that the factor(s) encoded by or present in NS1 RNA that stimulate mIgOCT expression are not general transcription factors present in a wide range of cell-types.

It is implicit in our interpretation of these results that approximately equal amounts of RF DNA are deposited in the nuclei of injected oocytes. In order to confirm that this was the case, DNA recovered from the same oocytes as those analysed by primer extension in fig.3 was analysed by gel electrophoresis followed by Southern transfer and hybridization with a radiolabelled M13mp18 probe. The results of this analysis (fig.4) show that similar amounts of injected DNA were present in the oocyte nuclei and that the DNA was recovered mainly in supercoiled form.

Although these experiments have been repeated it should be noted that not all batches of oocytes show the effects described above. The effects we report are consistent within batches of oocytes obtained on a single occasion from one female. However, some batches of oocytes (we estimate about half of them) show a relatively high level of expression of the mIgOCT DNAs even in the absence of injected mRNA. The reason for this variability is not known, but variability has been observed by other workers who have used oocytes as a system in which to analyse transcription and transcription factors (22, 23, 24).

DISCUSSION

Addition of octamer elements to a fragment of the Xenopus 68kdal albumin gene promoter has two main consequences. The activity of the promoter in oocytes can be stimulated by prior injection with myeloma cell mRNA, but the overall efficiency of the promoter is reduced. This reduction in efficiency cannot be explained by interruption of sequences required for albumin gene expression as the octamer elements are inserted at the boundary between the albumin promoter sequences and the vector sequences of the parent clone Δ-50. Hence the octamer is acting as a negative regulatory element. Control of immunoglobulin gene expression in mammals involves, inter alia, a positive regulatory mechanism and consistent with this is the observation that mutagenesis of the octamer leads to a decrease in expression of linked genes in B-cells, rather than an increase in the expression in other cell-types (11). However, in instances where an octamer has been inserted into a heterologous promoter, the activity of the promoter has not been assessed in cell-types in which it normally functions (10).

Xenopus histone H2B genes, which are transcribed in oocytes, contain octamer motifs,(30) so it is possible that oocytes contain a protein homologous to the ubiquitous octamer binding factor, NFA1/OTF1, which is apparently necessary for maximal transcription of the human histone H2B gene. This protein is apparently not able to activate promoters consisting only of an octamer motif linked to a heterologous promoter fragment that includes a TATA box, as such hybrid promoters are active in B-cells, but not in other cells which also contain the ubiquitous binding factor. If a similar protein is present in oocytes, then by binding to the octamer it may interfere with the assembly of transcription complexes on the albumin sequences of the mIgOCT promoters.

The activity of mIgOCT promoters is enhanced by prior injection of NS1 mRNA. We cannot say this induction is due to a single factor or to multiple factors. Neither can we say whether factor(s) is an RNA molecule, or a protein encoded by one of the mRNA species present. One obvious possibility is that the induction caused by NS1 mRNA is due to translation of an mRNA species encoding the B-cell-specific octamer binding factor NFA2/OTF2 (14,15). We believe that the experiments reported here have the important feature of directly assaying in vivo transcription, and so probably represent the activity of factors which do normally act in transcriptional activation of 'octamer' bearing promoters. Clearly the inducibility and the overall reduction in efficiency of the promoter is greater when two, rather than one, octamer sequences are present. Again we do not know the basis of this, but it is a common finding that sequences involved in regulation of transcription (particularly those within enhancers) exert a greater effect when present in multiple copies (31,32).

We now hope to use the oocyte assay system we have developed to clone cDNAs encoding the factor(s) required for tissue-specific expression of immunoglobulin genes.

ACKNOWLEDGEMENTS.

We are grateful to Dr. G. Ryffel for the provision of a Xenopus albumin clone. We thank Mrs. G. Scott for synthesising the oligonucleotides used in these experiments. This work was funded by the Science and Engineering Research Council.

REFERENCES

1. Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983), Cell 33, 717-728.
2. Banerji,J., Olson,L. and Schaffner,W. (1983), Cell 33, 729-740.
3. Neuberger,M.S. (1983), EMBO.J. 2, 1373-1378.
4. Mason,J.O., Williams,G.T. and Neuberger,M.S. Cell 41, 479-487.
5. Grosschedl,R. and Baltimore,D. (1985), Cell 41, 885-897.
6. Falkner, F.G. and Zachau, H.G. (1984), Nature 310, 71-74.
7. Sive,H.L., Heintz,N. and Roeder,R.G. (1986), Mol.Cell.Biol. 6, 3329-3340.
8. Murphy,J.T., Burgess,R.R., Dahlberg,J.E. and Lund,E. (1982), Cell 29, 265-274.
9. Ballard,D.W. and Bothwell,A. (1986), Proc.Natl.Acad.Sci. USA, 83, 9626-9630.
10. Dreyfus,M., Doyen,N. and Rougeon,F. (1987), EMBO.J. 6, 1685-1690.
11. Wirth,T., Staudt,L. and Baltimore,D. (1987), Nature 329, 174-178.
12. Singh,H., Sen,R., Baltimore,D. and Sharp,P.A. (1986), Nature 323, 154-158.

13. Fletcher,C., Heintz,N. and Roeder,R. (1987), Cell 51, 773-781.
14. Staudt,L.M., Singh,H., Sen,R., Wirth,T., Sharp,P.A. and Baltimore,D. (1986), Nature 319, 640-643.
15. Scheidereit,C., Heguy,A. and Roeder,R.G. (1987), Cell 51, 783-793.
16. Mous,J., Stunnenberg,H., Georgiev,O. and Birnstiel,M.L. (1985), Mol.Cell.Biol. 5, 2764-2769.
17. Maxson,R., Ito,M., Balcells, S., Thayer,M. and Etkin,L. (1987) In Firtel,R.A. and Davidson,E.H.(eds), Molecular approaches to developmental biology, Alan R.Liss, Inc, pp.253-265.
18. Wigley,P.L. and Wells,J.R.E. (1987), Mol.Cell.Biol. 7, 3853-3856.
19. Masu, Y., Nakayama,K., Tamaki,H., Marada,Y., Kuno,M. and Nakanish,S. (1987), Nature 329, 836-838.
20. Sweeney,G., Brooks,A., Day,P and Old,R. (1987) Nucl.Acids.Res. 15, 5889.
21. Old,R.W., Sheikh,S.A., Chambers,A., Newton,C.A., Mohammed,A. and Aldridge,T.C. (1985), Nucl.Acids.Res., 13, 7341-7358.
22 Kohler,G., Howe,S.C. and Milstein,C. (1976) Eur.J.Immunol. 6, 292-295.
23. Aviv,H. and Leder,P. (1972), Proc.Natl.Acad.Sci. USA 69, 1408-1412.
24. Chirgwin,J.M., Pryzbula,A.E., McDonald,R.J. and Rutter,W.J. (1979) Biochemistry, 18, 5294-5299.
25. Mertz,J. and Gurdon,J.B. (1977) Proc.Natl.Acad.Sci. USA, 74, 1502-1506.
26. Maniatis,T., Fritsch,E.F. and Sambrook,J. in Molecular Cloning (Cold Spring Harbour Laboratory, New York, 1982).
27. Korn,L.J. and Gurdon,J.B. (1981), Nature 289, 461-465.
28. Knowland,J., Theulaz,I., Wright,C.V.E. and Wahli,W. (1984) Proc.Natl.Acad.Sci. USA 81, 5777-5781.
29. Bendig,M.M. and Williams,J.F. (1984), Mol.Cell.Biol. 4, 2109-2119.
30. Perry,M., Thomsen,G.H. and Roeder,R.G. (1985), J.Mol.Biol. 185, 479-499.
31. Herr,W. and Gluzman,Y. (1985), Nature 313, 711-714.
32. Gerster,T., Matthias,P., Thali,M., Jiricng,J. and Schaffner,W. (1987), EMBO.J. 5, 1323-1330.