A more efficient and specific strategy in the ablation of mRNA in *Xenopus laevis* using mixtures of antisense oligos

Richard Morgan, Michael Edge¹ and Alan Colman*

School of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT and

¹Cancer Research Department, Zeneca Pharmaceuticals, Mereside Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Received May 11, 1993; Revised and Accepted August 16, 1993

ABSTRACT

Previously, antisense oligodeoxyribonucleotides (oligos) have been used to ablate specific mRNAs from the maternal RNA pool of *Xenopus laevis* oocytes. However, this strategy is limited by the dose of oligo which can be used and the fact that 100% cleavage of the target RNA is rare. Further, non-specific cleavage of other RNAs can also occur. We demonstrate that the use of several oligos against the histone H4 RNA results in a marked improvement in the efficiency of target degradation, due to synergistic action between oligos and the existence of RNA in at least two different secondary structures. We show, by using a set of overlapping oligos complementary to the entire H4 RNA, that the amount of oligo required for efficient target ablation is greatly lowered and non-specific effects are reduced.

INTRODUCTION

Antisense oligodeoxynucleotides (oligos) have proved useful for inhibiting gene expression in a range of systems (for reviews, see Colman [1] and Uhlmann and Peymann [2]). Studies in *Xenopus laevis* oocytes and embryos have the particular advantage that the effect of the oligo on its target RNA can be directly assessed [3, 4]. It has been shown that oligos containing phosphodiester or phosphorothioate linkages cause RNA cleavage and that this effect is mediated by RNase H, a nuclease which is specific for the RNA portion of DNA-RNA hybrids. The injection of antisense oligos into oocytes is an effective and practical way of removing a maternal mRNA of choice from the RNA pool, which in turn is useful in studying the role of a given message during oogenesis and the early stages of development [4,8].

In general, and certainly in oocytes specifically, antisense oligo mediated ablation of an RNA target suffers from several drawbacks. Firstly, non specific targeting of other messages is seen, with detrimental consequences for the cell [5–9]. This is probably due to shorter regions of complementary sequence

between the oligo and other RNAs, allowing hybrids to form which are also substrates for RNase H. Secondly, a single oligo is rarely entirely effective in ablating all of its target [4,10], especially in the case of relatively abundant RNAs, even when higher oligo concentrations or repeated exposure to oligos are used. Third, the concentrations of oligo needed to ablate a significant portion of a specific RNA population can be toxic to the cell [4]. Finally, there is considerable variation in the effectiveness of oligos aimed at different regions of the RNA [10], and selection of the most effective oligo has remained an empirical exercise.

Solutions to some of the above problems have been tried using modified, nuclease-resistant oligos [7,8,10]. The work reported in this paper addresses the issues of oligo selection and efficacy. Our original strategy involved the *in vitro* ablation of histone H4 RNA in the presence of a random set of 10-mer oligos together with RNase H. We reasoned that as the oligo concentration was lowered, a situation might arise where cleavage was mediated by one specific oligo which was complementary to a particularly accessible RNA site. Although direct experimentation showed this to be incorrect, with no one oligo being identifiable, we did find that the effective, total oligo concentration needed, was greatly reduced. Further reductions in oligo concentrations were possible when, instead of a random oligo mixture, a set of oligos complementary to the entire histone H4 RNA were used. In this paper we provide evidence that the improved efficacy of oligo ablation is a consequence of cooperative interactions between different oligos and the existence of H4 RNAs with different secondary structures.

METHODS

Oligo synthesis

Oligos were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by reverse phase HPLC. Oligo sequences are: h-1, TAGATGAGAC CTGAGATGCG; h-2, TACACCACAT CCATGGCGGT; h-3, ATGCGCTTGA CTCCCCCTCT; h-4, TGGCGGTAAC AGTCTTCCTCT and h-5,

* To whom correspondence should be addressed

AGCGGTAGAG AGTGCGGCC. A random 10 mer was generated by supplying equimolar concentrations of all four phosphoramidites (Cruachem, U.K.).

RNA transcription

H4 RNA, both sense and antisense, was transcribed from the linearised plasmids pSP64-H4 and pSP65-H4 respectively; these plasmids contain a 750 base pair (bp) insert of histone H4 (H4) DNA. Antisense RNA for probes against mRNA encoding the $\beta 3$ subunit of Na,K ATPase was transcribed from a linearised pGEM2 plasmid containing a 1450 bp cDNA insert. Antisense Vg1 RNA, also for probing northern blots, was transcribed from a linearised Blue script plasmid containing a 2.5 kbp cDNA insert. 5 μ g of DNA were incubated at 37°C for one hour in 10 μ g/ml BSA, 40mM Tris HCl pH 7.9, 6mM MgCl₂, 10mM dithiothreitol, 2mM spermidine, 40U of SP6 polymerase and 20U of human placental RNase inhibitor (both from Pharmacia) in a total volume of 50 μ l. For labelled RNA used in the *in vitro* assay (see below), 500 μ M of each rNTP and 0.5 μ Ci of α^{32} P CTP (3000Ci/mmol, Amersham, U.K.) were used in the transcription mixture. For hybridisation probes, 500 μ M of ATP, GTP and UTP, 5 μ M of CTP and 5 μ Ci of α^{32} P CTP (3000Ci/mmol, Amersham, U.K.) were used in the transcription.

The reaction was processed as follows; the DNA template was removed by incubating for a further ten minutes at 37°C with 7.5U of DNase I (Pharmacia, U.S.A.). RNA was then recovered by phenol:chloroform extraction and ethanol precipitation. The quantity of RNA produced was determined by spotting part of the transcription mix on DE81 paper, half of which was allowed to air dry and the other half washed as follows: two, five minute washes in 0.15M Na₂HPO₄ and one, five minute wash in each of water and acetone: methanol (1:1). After drying, both washed and unwashed filters were placed in 1ml of Optiphase High Safe scintillation fluid (Fisons, U.K.) and counted for five minutes. The percentage incorporation of label into the RNA, and hence the amount of RNA produced, was calculated from the difference in counts between the washed and unwashed filters. Finally, the RNA was resuspended in water at 50ng/ μ l.

In vitro assay for oligo mediated RNA cleavage

This method is modified after Baker et al. [10]. 50ng of radiolabelled RNA were incubated for one hour at 21°C in 20 μ l of 100mM KCl, 20mM Tris HCl pH 7.4, 1.5mM MgCl₂, 1mM dithiothreitol and 50 μ g/ml BSA, 0.2U RNaseH (Pharmacia, U.S.A.) and 0.5U of human placental RNase inhibitor (Pharmacia, U.S.A.). The reaction was stopped by phenol:chloroform extraction followed by ethanol precipitation. For sequential digestions, reactions were stopped after one hour, again by phenol:chloroform extraction. The oligo was removed by LiCl precipitation (0.1 vols of 8M LiCl, then incubate on ice for two hours), followed by ethanol/NH₄Ac precipitation to remove any remaining LiCl. Pellets were taken up in 6 μ l water and the second reaction started in the same way as for the first, the RNA being re-extracted and precipitated after a further hour of incubation. The recovered RNA was taken up in loading buffer (20mM EDTA, 1 $\times$ MOPS [3-(N-Morpholino) propane sulphonic acid], 80% formamide and 0.01% bromophenol blue) and heat denatured. Samples were run on a 2% agarose-MOPS-15% formaldehyde gel at 5V/cm for two hours, followed by fixing, drying and exposure to autoradiography film.

Injection of stage VI oocytes

Stage VI *Xenopus laevis* oocytes, as defined by Dumont [11], were injected with oligos or M13 digest in 50nl of water. Injected oocytes were incubated for two hours at 21°C in modified Barths saline [3].

Extraction of RNA and Northern blotting

Oocytes were homogenised in a buffer containing proteinase K (Sigma, U.S.A.), and total nucleic acids were extracted and precipitated as described by Krieg and Melton [12]. The RNA pellet was taken up in water to give a concentration of 2mg/ml and 4 μ g of each RNA sample were run on a MOPS-formaldehyde gel after heating in an equal volume of running buffer (see above). RNA was blotted onto a nitrocellulose membrane (as described by the manufacturers - Amersham) and baked at 80°C for two hours. The filter was probed with anti-H4, anti-Vg1 or anti- $\beta 3$ RNA, washed as previously described [13], and exposed to X-ray film.

Cloning of H4 into M13

An H4 DNA fragment was cut from pSP64-H4 and ligated into M13mp18 and this was transfected into XL1 cells [13]. Plaques were screened by extraction of the RF DNA and subsequent restriction analysis. Single stranded DNA was prepared from the culture supernatant and taken up, after ethanol precipitation, in water at 1 mg/ml.

Digestion of H4-containing single stranded M13 by DNase I

Single stranded M13(H4) was digested at 37°C in 40mM Tris HCl pH 7.4, 6mM MgCl₂, 0.5 mg/ml DNA and 10U/ml DNaseI (Pharmacia, U.S.A.) for ten minutes at 37°C. A 1 μ l sample of the reaction mixture was end labelled with polynucleotide kinase (Pharmacia, U.S.A.) using the protocol for the exchange reaction as described by the manufacturer. The labelling reaction was stopped by the addition of an equal volume of loading buffer (80% formamide, 20mM EDTA pH8.0 and 0.05% w/v bromophenol blue and xylene cyanol). Products were resolved by running on a 8M urea, 20% acrylamide gel, 30 cm in length, at 20W, until the lower dye front reached the end. After fixing and drying the products were visualised by exposing to X-ray film.

RESULTS

The effect of anti-H4 RNA oligos varies with sequence

Oligos aimed at different parts of a target RNA can have dramatically different efficiencies at mediating RNase H-dependent cleavage, as shown previously for histone H4 RNA [3,4,10], and further demonstrated in the experiment shown in fig 1 where five different anti-H4 oligos are tested. Each oligo is present in saturating concentrations and further cleavage of the residual RNA cannot be achieved even when the treated RNA is repurified and incubated again with fresh enzyme and oligo (data not shown—but see fig 2, cf lanes 6 and 7). The differential effect of the oligos is thought to be a result of the relative accessibility of different regions of the RNA to each oligo. Such differences could be due to the secondary structure adopted by the RNA, or to protein binding [14], both of which could mask potential oligo binding sites. The latter explanation is no longer favoured as similar relative efficiencies for cleavage by a group of different anti-H4 oligos are seen both *in vivo* and with purified

Figure 1. The relative efficiencies of oligos aimed at different regions of the same target RNA. **A:** The locations of the target sites for oligos h-1 to h-5 on H4 RNA. **B:** Anti H4 oligos h-1 to h-5 were incubated with 50ng of radiolabelled sense H4 RNA (i.e. 2.5µg/ml) in the presence of RNase H as described in methods. RNA was then extracted and run on a 2% formaldehyde-agarose gel and this was subsequently fixed, dried and autoradiographed. The total concentration of oligo in each reaction was 50µg/ml, so for example in reaction six above, each individual oligo was present at 10µg/ml.

Figure 2. Sequential or joint incubations of H4 RNA with antisense oligos. Labelled H4 RNA was incubated with oligos h-3 and h-4 either sequentially (lanes 3-6) or together (lane 2), in the presence of RNaseH. For sequential reactions, the first digestion was with an oligo at 50µg/ml, followed by phenol-chloroform extraction and LiCl precipitation to remove the oligo and recover the RNA which was then digested with a second oligo, also at 50µg/ml. The order in which these reactions were done are indicated in the figure. For the joint digestion, oligos h-3 and h-4 were both present at 25µg/ml.

oocyte RNA *in vitro* [10]. Further, all oligos cleave with more or less the same efficiency when they are pre annealed to RNA after treatment to destroy native secondary structure (data not shown). This suggests that there is no difference in the intrinsic susceptibility of different hybrids. Thus it seems probable that RNA secondary structure is mainly, if not entirely, responsible for determining the relative efficiency of oligo-mediated cleavage.

Figure 3. Northern analysis of RNA extracted from oocytes injected with oligos h-3, h-4 or h-1, h-2 and h-3 or all five in combination. All oocytes were injected with 50nl of water containing oligo at a total concentration of 0.1mg/ml. Thus when all five are injected together, the concentration of each oligo in the mixture was 0.02mg/ml. Subsequently RNA was extracted and probed for either (A) histone H4 RNA or (B) Vg1 RNA. Densitometric readings indicate the reduction of full length H4 RNA was 78% (lane 1), 42% (lane 2), 9% (lane 3) and ~100% (lane 4). The difference between the observed *in vivo* and *in vitro* cleavage patterns (see fig 1) for oligos h-3 and h-4 is due to the rapid cleavage of the 3' fragments *in vivo* [3].

It is also clear from fig 1 that a cocktail of three of the oligos is more effective at mRNA ablation than each oligo individually (cf lane 7 with lanes 1,2 and 5).

The mechanism of enhanced RNA cleavage

Two possible explanations can be envisaged as to why a mixture of oligos is more effective in cleaving H4 RNA. Firstly, the RNA could exist in two or more different secondary structures. Since it seems that secondary structure is the major determinant in oligo cleavage efficiency, a mixture of oligos may be required to cause ablation of the different structures present. Alternatively, or additionally, oligos may act cooperatively by altering the secondary structure of the RNA to which they bind, thus allowing easier access to other oligos aimed at different sites. In order to try to distinguish between these possibilities we tried digesting RNA with two different oligos, h-3 and h-4, either consecutively or concurrently. The results are shown in fig 2. Cleavage with one oligo followed by LiCl precipitation to remove it from the RNA and incubation with the second results in a mixture of products in both cases. For example, from lane 4 it is clear that the second oligo, h-3, makes a further contribution to cutting the RNA after digestion with oligo h-4. The sizes of the additional products correspond to the cutting of full length RNA (comparing lanes 4, 5, and 6). Hence the second oligo, h-3, can cut RNA molecules unaffected by exposure to oligo h-4, indicating that

Figure 4. The generation of a set of anti H4 oligos. Single stranded M13 containing H4 antisense DNA was digested with DNase I to give a range of different sized products. Digestion products were end labelled with 32 P and resolved on a 20% acrylamide gel. The markers are end labelled oligos of sizes 20, 22 and 25 bases.

Figure 5. *In vitro* cleavage by RNase H of histone H4 RNA mediated by M13-H4 DNA fragments. Lanes 1 and 2; histone sense RNA (sRNA) and antisense RNA (aRNA) respectively, incubated with RNase H only. Lane 3, H4 sense RNA incubated with fragments of single stranded M13 lacking the H4 DNA insert, the total oligo concentration was 50 μ g/ml. Lane 4, the M13-H4 digest incubated with antisense H4 RNA. Lane 5, sense H4 RNA incubated with M13-H4 DNA digest at a concentration of 5 μ g/ml. Lane 6, incubation with M13-histone 4 digest at 50 μ g/ml. Lane 7, incubation of histone H4 sense RNA with oligo h-3 at 5 μ g/ml.

these RNA molecules have a different secondary structure. When RNA is incubated with both oligos simultaneously, there is a further reduction in the amount of full length surviving transcript (lane 2). More significantly, however, the size of the observed cleavage products corresponds to secondary cutting of the original products, something not seen in consecutive digestions. This implies that cooperative interactions must also occur in the oligo mediated degradation of H4 RNA. We conclude that both multiple secondary structures and cooperativity between different oligos are contributory factors in enhanced RNA cleavage.

A complete oligo set covering the entire histone H4 RNA is extremely effective at ablating its target

The success of mixing oligos aimed at different targets on the same RNA is clear *in vitro*. For example, the mixture of all five anti-H4 oligos are far more effective in mediating the cleavage

Figure 6. The *in vivo* effects of oligos h-3, h-4, h-5 or of the anti-H4 oligo set generated by M13-H4 digestion. Northern analysis of RNA extracted from oocytes injected with either a single anti histone H4 oligo (50nl at 0.1mg/ml, lanes 3–5) or with M13-antisense H4 DNA digest (50nl at 0.05mg/ml, lane 1). As a control on non specific degradation the filter was also probed with antisense RNA to β 3 Na,K ATPase message and to Vg1 RNA.

of H4 RNA (fig 1, lane 6) than are any of the individual oligos in this mixture (lanes 1–5). This is true also *in vivo* (fig 3). In this experiment, *Xenopus* oocytes were injected with different oligos singly or in combination. Clearly the combination was most effective (cf lane 4 with lanes 1–3).

Could this effect be improved still further to allow complete ablation *in vivo* using a significantly lower dose of oligo? If, as we suggest, RNA exists in multiple secondary forms and oligo binding can influence the structure at distal sites, the best strategy may be to use a series of oligos all aimed at different regions of the target RNA. Instead of synthesizing oligos separately for the entire length of the RNA we used limited digestion of antisense single stranded H4 DNA by DNase I. Along with practical considerations, there is another advantage over using a large set of oligos. The efficiency of oligo-mediated cleavage can change quite considerably if the target site is shifted by only a few bases on the RNA. For example, in fig1, oligos h-2 and h-4 have a ten base overlap and yet of the two, h-4 brings about considerably more cleavage. The use of a set of fragments randomly generated from a complete antisense H4 DNA should ensure that all permutations of 'oligo' will be present. This mixture was generated as described below.

The 750 base pair DNA H4 fragment was cloned into M13mp18, and the single stranded form of this was recovered from the supernatant of an over night culture. 100 μ g were digested with DNase I to give a ladder of different sized products (fig 4). The sizes ranged from a few bases to about 100, but most of the fragments are 18–23 bases long. Ten bases has previously been identified as the minimum size of oligo which can mediate cleavage in *Xenopus* oocytes [3]. Testing the M13-H4 digest mixture *in vitro* (fig 5) shows that the ablation of target RNA is increased considerably. At 5 μ g/ml (final concentration) the mixture still achieves 100% ablation (lane 5); the best single oligo (h-3, see figs 1 and 3) in lane 7, does not mediate any significant cleavage at this concentration. To test for non-specific effects *in vitro*, the M13-H4 digest was incubated with antisense H4 RNA (lane 4), but no cleavage was observed. Further, no cleavage is observed when M13 digest containing no H4 insert is used

(lane 3). Incubation of H4 RNA with undigested M13-H4 DNA at 50 μ g/ml also fails to mediate cleavage by RNase H (data not shown).

The M13-H4 digest was tested *in vivo* by injecting into oocytes along with oligos h-3, h-4 and h-5 (fig 6). The oligos were injected at 0.1mg/ml, whereas the digest was injected at 0.05mg/ml. Injection of the M13-H4 digest at this concentration results in total ablation of the target (lane 1), whilst the single oligos injected at a ten-fold higher concentration cause only limited cleavage. As a control, the filter was also probed for RNA encoding the β 3 subunit of Na,K ATPase [16] and Vg1 RNA [17]. Vg1 mRNA was unaffected by any of these treatments. β 3 RNA was unaffected by the M13 digest but was, unexpectedly, partly degraded by oligo h-5. However H4 and β 3 RNA share a ten base, GC-rich region which is complementary to h-5 and this is probably responsible for the observed degradation. Presumably the M13-H4 digest also contains a proportion of fragments complementary to this RNA, however they would be at a much lower concentration.

DISCUSSION

Recently, concern has been growing that the use of antisense oligos in a range of different systems, but particularly *Xenopus* oocytes and embryos, causes detrimental non-specific effects on the cell. This could be a result of both an imbalance in the intracellular nucleotide pool after oligo breakdown and of non-specific cleavage of RNAs other than the intended target. Non-specific effects probably occur as a result of short regions of sequence complementarity between the injected oligo and non-target RNAs acting as substrates for RNase H [5,6,18,19].

We show that a mixture of oligos is more effective in cleaving target RNA, both *in vivo* and *in vitro*, than any of the individual oligos represented in the mix. In order to try to optimise this effect we have made a set of oligos aimed at random parts of the target RNA and covering its entire length in total. This was achieved by limited DNase I digestion of single stranded, antisense, H4 DNA contained within M13 vector. The majority of the resulting oligos are between 18 and 23 bases long. Use of this digest greatly enhances the process of RNase H mediated degradation. This observation is supported by that of Minshall and Hunt [20], who reported that HaeIII fragmentation of antisense DNA cloned into M13 considerably enhances its effect in mediating hybrid arrest of translation.

The dramatic increase in efficiency of RNA cleavage when using a large set of oligos is explained, at least in part, by the existence of the target RNA in more than one secondary conformation. This is shown well in fig 2, the sequential digestion of RNA by oligo h-3 followed by h-4, or vice-versa, results in additional cleavage of the target RNA, something which is not observed with two digestions by the same oligo (compare lanes 3 and 4 to 5 and 6). Further there is an additional reduction in the amount of surviving full length transcript. The incubation of oligos h-3 and h-4 together results in additional, secondary cleavage products corresponding to the recleavage of the initial products. As this does not happen when oligos are used separately, we infer that in this case cooperative interaction does occur between oligos. Whether this actually quantitatively affects the amount of surviving full length transcript is unclear, but it seems probable that it could. It is likely however that the use of a mixture of oligos has a greater effect than any single oligo as a result of the target RNA existing in at least two, but probably

multiple secondary forms. The increase in efficiency has meant that a lower dose need be used to successfully knock out histone mRNA, an abundant message. This effect has been maximised by using a set of oligos aimed at different sites across the length of the target RNA, generated by DNase I digestion of full length, single stranded, antisense H4 DNA.

Will this help reduce non specific effects? As less oligo is being injected, there will be less of a disturbance to the nucleotide pool. However, with a large number of different oligos, one might expect the chance of there being a significant match between one of them and a non-target RNA increases considerably. This, however, does not appear to be the case. Whilst the injection of the M13-H4 digest caused total ablation of the histone 4 message in the oocyte, it left intact the two endogenous, non-target RNAs for which we tested (i.e Vg1 and the RNA encoding the β 3 subunit of the Na,K-ATPase unit). In contrast one of the anti-H4 oligos does ablate β 3 message after injection into oocytes (fig 6). This is not entirely surprising as it has a ten base sequence complementary to β 3 RNA. The M13-H4 digest oligos will also contain this sequence as it was formed by limited, random digestion of antisense H4 DNA. This suggests that this sequence is at too low a concentration in the oligo mix to ablate the control. It seems likely then that the use of such oligo mixtures could actually reduce non-specific effects by lowering the concentration of individual oligos to a point where the binding of non-target sequences becomes a very rare event.

A further refinement of this strategy would be to purify the single stranded, antisense DNA from the M13 vector prior to DNaseI digestion, thus eliminating the non-targeting oligos from the mix. Single stranded DNA could be cut by annealing an oligo to the appropriate restriction site thus making it double stranded. This could increase yet further the efficiency and specificity of RNaseH mediated cleavage of the RNA.

The observations above might explain why our initial experiments involving the use of a mixture of random oligos failed to provide a screen for particularly sensitive sites in the H4 mRNA (data not shown); presumably with low concentrations of the mixture the concentration of any 'efficient' oligo would be grossly suboptimal, whilst at high mixture concentrations the concerted action of many complementary oligos would mask the effect of particularly 'efficient' oligos.

It has been known for some time that a major problem in the development of unmodified oligos as therapeutic reagents is their vulnerability to nucleases, especially those in the blood [7, 21-23]. Although this nuclease sensitivity is presumably compensated for by the increased efficiency shown by the M13-H4 digest approach in oocytes, the therapeutic use of unmodified oligos will be further complicated by serum-mediated degradation and the impermeability of the plasma membrane of the cells in tissues. This has led to the development of modified oligos which show increased nuclease resistance and improved permeability properties (see Toulme [24], for a comprehensive review). Such oligos have been used in both frog oocytes [7,8] and cultured mammalian cells [24]. Oligos with phosphorothioate or amidate modified linkages at their 3' and 5' ends can resist attack by endonucleases whilst still forming RNase H cleavable hybrids with RNA by virtue of unmodified central linkages. Indeed Tidd [25] showed, *in vitro*, that oligos protected in this way with methyl phosphonate linkages can increase specificity by reducing cleavage resulting from non-specific hybrid formation. The method of limited digestion of full length antisense DNA outlined here could not be used with modified DNA. We

have shown, however, that the efficiency of antisense knock-out can be improved even by using only a small group of oligos. The way ahead now may be to make use of modified oligos and the cooperative interactions which may occur between them in order to develop a strategy which allows effective and specific knock-out of a target RNA at a lower dose than has so far proved possible.

ACKNOWLEDGEMENTS

We are grateful to Dr David Holland of Zeneca Pharmaceuticals who supplied most of the oligos used in this study. R.M. was supported by a SERC-CASE studentship, and A.C. acknowledges the support of the Wellcome Trust.

REFERENCES

- Colman, A. (1990) *J. Cell Sci.* **97**, 399–409.
- Uhlmann, E. and Peyman, A. (1990) *Chem. Rev.* **90**, 543–584.
- Shuttleworth, J. and Colman, A. (1988) *EMBO J.* **7**, 427–434.
- Shuttleworth, J., Matthews, G., Dale, L., Baker, C. and Colman, A. (1988) *Gene* **72**, 267–275.
- Woolf, T.M., Melton, D.A. and Jennings, C.G.B. (1992) *Proc. Natl. Acad. Sci., USA* **89**, 7305–7309.
- Smith, R.C., Bement, W.M., Dersch, M.A., Dworkin-Rastl, E., Dworkin, M.B. and Capco, D.G. (1990) *Development* **110**, 769–779.
- Woolf, T.M., Jennings, C., Rebagliati, M.R. and Melton, D.A. (1990) *Nucleic Acids Res.* **18**, 1763–1769.
- Dagle, J.M., Walder, J.A. and Weeks, D.C. (1990) *Nucleic Acids Res.* **18**, 4751–4757.
- Dash, P., Loton, A., Knapp, M., Kandel, E. and Goelet, P. (1987) *Proc. Natl. Sci., USA* **84** 7869–7900.
- Baker, C., Holland, D., Edge, M. and Colman, A. (1990) *Nucleic Acids Res.* **18**, 3537–3543.
- Dumont, J.N. (1972) *J. Morphol.* **136**, 153–180.
- Krieg, P. and Melton, D.A. (1984) *Nucleic Acids Res.* **12**, 7057–7070.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (2nd edition). Cold Spring Harbour University Press, Cold Spring Harbour.
- Marello, K., LaRovere, J. and Sommerville, J. (1992) *Nucleic Acids Res.* **20**, 5593–5600.
- Marcus-Sekura, C.J., Woerner, A.M., Shinozuka, K., Zon, G. and Quinnan, G.V. (1987) *Nucleic Acids Res.* **15**, 5749–5763.
- Good, J.P., Richter, K. and Dawid, I.B. (1990) *Proc. Natl. Acad. Sci., USA* **87**, 9088–9092.
- Weeks, D.L. and Melton, D.A. (1987) *Cell* **51**, 861–867.
- Bacon, T.A. and Wickstrom, E. (1991) *Oncogene Res.* **6**, 13–19.
- Storey, A., Oates, D., Banks, L., Crawford, L. and Crook, T. (1991) *Nucleic Acids Res.* **19**, 4109–4114.
- Minshall, J. and Hunt, T. (1986) *Nucleic Acids Res.* **14**, 6433–6451.
- Cazenave, C., Loreau, N., Thuong, N.T., Toulme, J.-J. and Helene, C. (1987) *Nucleic Acids Res.* **15**, 4717–4736.
- Wickstrom, E. (1986) *J. Biochem. and Biophys. Methods* **13**, 97–102.
- Rebagliati, M.R. and Melton, D.A. (1987) *Cell* **48**, 599–605.
- Toulme, J.-J. in (1992) 'Antisense RNA and DNA' Murray, J.A., ed., Wiley & Sons, New York 175–194.
- Giles, R.V. and Tidd, D.M. (1992) *Nucleic Acids Res.* **20**, 763–770.