Reverse transcription of phage RNA and its fragment directed by synthetic heteropolymeric primers

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ABSTRACT

DNA synthesis catalysed by RNA-directed DNA-polymerase (reverse transcriptase) was found to proceed on the RNA template of an MS2 phage in the presence of heteropolymeric synthetic octa- and nonadeoxyribonucleotide primers complementary to the intercistronic region (coat protein binding site) and the region of the coat protein cistron, respectively. The product of synthesis consists of discrete DNA fractions of different length, including transcripts longer than 1,000 nucleotides. The coat protein inhibits DNA synthesis if it is initiated at its binding site, but has no effect on DNA synthesis initiated at the coat protein cistron. It has been suggested that, in this system, the initiation of DNA synthesis by synthetic primers is topographically specific. The MS2 coat protein binding site (an RNA fragment of 59 nucleotides) serves as a template for polydeoxyribonucleotide synthesis in the presence of octanucleotide primer and reverse transcriptase. The product of synthesis is homogenous and its length corresponds to the length of the template. The effective and complete copying of the fragment having a distinct secondary structure proves that the secondary structure does not interfere, in principle, with RNA being a template in the system of reverse transcription.

INTRODUCTION

RNA-directed DNA-polymerase catalyses DNA synthesis on various templates like RNA of oncornaviruses ^{1,2}, animal ^{3,4}, bacterial ⁵, and plant ⁶ viruses, as well as poly(A)-containing messenger RNAs ^{7,8}, and polyadenylated ribosomal RNAs ⁹, i.e. it is not selective with respect to the nature of a template. The enzyme is not selective either to a primer which can be a short homopolymer of the deoxyribo series (oligo(dT), oligo(dG), etc.), a heteropolymer of the ribo series (tRNA^{Try}, tRNA^{Pro}), or a heteropolymer of the deoxyribo series, for example a synthetic heterooctadeoxyribonucleotide (for ref. see ⁷). The absence of the template-primer specificity makes

reverse transcriptase a universal tool capable of synthesizing almost any DNA, so far as the regions of a template and a primer are complementary.

It seems highly desirable therefore to find a general approach that would be helpful to bring forth reverse transcription starting from a preset site on a template, and to obtain in this way DNA fragments required for sequence analysis and many other purposes.

As we have shown earlier ⁵, an octadeoxyribonucleotide, complementary to the spacer region between the coat protein and the replicase cistrons in a R17 phage, can initiate reverse transcription of R17 RNA catalysed by reverse transcriptase isolated from avian myeloblastosis virus. The product thus formed and the R17 RNA which served as a template are complementary, and the synthesis of the product depends entirely on the presence of a primer.

The present work was done in order to study the selectivity of initiation of DNA synthesis since it is possible that, in the case of a relatively short synthetic primer and a sufficiently long template, more than one region on a template can be, either entirely or partly, complementary to a primer.

MATERIALS AND METHODS

The preparation of reverse transcriptase was isolated from a purified avian myeloblastosis virus according to Kacian et al. ¹⁰ with some modifications ¹¹, RNA of phages R17, MS2, f2 and Q β was isolated as described by Berzin et al. ¹². The MS2 R(-53 $\rightarrow$ 6) fragment of RNA, protected with the coat protein from hydrolysis by T₁ RNase, was isolated according to Berzin et al. ¹³, and the coat protein, according to Sugiyama et al. ¹⁴. A primer, an octadeoxyribonucleotide d(G-G-T-A-A-T-G-G), was synthesized as described by Metelev et al. ⁵. A nona-deoxyribonucleotide was prepared by the following scheme. Abbreviations used here are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature (1970, J. Biol. Chem. 245, 5171-5176):

$I.H^+ 2.d(MeOTr)anC-anC-bzAp; TPS \rightarrow d(MeOTr)anC-anC-bzA-T-anC-$
 $-T-T-T-T(iBu) \xrightarrow{I.NH_3} 2.H^+ \rightarrow d(C-C-A-T-C-T-T-T-T) \text{ Yield 17 per cent}$
 $d(MeOTr)T-anC$ and $d(MeOTr)anC-anC-bzA$ were synthesized as described by Weber and Khorana¹⁵. The synthesis of $d(T-T-T-T)(iBu)$ is described earlier¹⁶.

Synthesis of 3'-phosphates of $d(MeOTr)T-anCp$ and $d(MeOTr)anC-anC-bzAp$ oligonucleotides: 5'-O-methoxytrityl derivatives of dinucleoside phosphate and trinucleoside phosphate were phosphorylated in pyridine by β -cyanoethyl phosphate, taken in a 3-5-fold excess, in the presence of TPS (1.2 mmol per 1 equivalent of phosphate in the reaction mixture; 20°C; 6-7 hrs); 3'-phosphates of oligonucleotides were isolated by extraction¹⁶. $d(MeOTr)T-anC-T-T-T-T(iBu)$ was prepared from 2.74 g (2.3 mmol) of $d(MeOTr)T-anCp$ and 2 g (1.0 mmol) of $d(T-T-T-T)(iBu)$ using 3 g (10 mmol) of TPS (20°C; 5.5 hrs). The product was isolated by ion exchange chromatography on DEAE-cellulose (2.5 x 60 cm; HCO_3^-) in a linear TEAB concentration gradient. $d(MeOTr)anC-anC-bzA-T-anC-T-T-T-T(iBu)$ was prepared in a similar way from 80 mg (0.036 mmol) of $d(MeOTr)anC-anC-bzAp$ and 23.2 mg (0.009 mmol) of $d(T-anC-T-T-T-T)(iBu)$ using 66 mg (0.216 mmol) of TPS (20°C; 4 hrs). The product was isolated by ion exchange chromatography on DEAE-cellulose (1.8 x 33 cm; HCO_3^-). $d(C-C-A-T-C-T-T-T-T)$ was obtained after the elimination of protecting groups¹⁵, and the product was purified by ion exchange chromatography on DEAE-cellulose in the Tomlinson-Tener system at pH 7.0 and rechromatography in the same system at pH 3.5. Spectral characteristics: $\lambda_{max} = 267 \text{ nm}$; $\lambda_{min} = 237 \text{ nm}$; $E_{260/280} = 1.37$ (the theoretical value is 1.45). The ratio between the products of the nonanucleotide hydrolysis by PDE of snake venom is: $dC:dpA-dpC:dpT = 1.3:1:2:5.25$.

The concentration of components (in mM) in the complex formation of the MS2 RNA with the coat protein was as follows (total volume 0.1 ml): Tris-HCl (pH 7.5), 50; $MgCl_2$, 10; KCl, 80 (TMK buffer); MS2 [^{32}P]RNA, 0.61 mg/ml, specific activity 400 cpm/ μ g; coat protein of MS2 phage, 12.5 nmol/ml (the molar ratio between the coat protein and MS2 RNA was 22.4). The mixture was incubated 10 min at 0°C, diluted with 1 ml of cold

TMK buffer, passed through a nitrocellulose filter (Schleicher & Schuell, B-6), washed twice with 1 ml of TMK buffer, dried, and the radioactivity was then determined in a toluene-scintillation counter. The effectiveness of binding was 30-35 per cent.

The composition of the incubation mixture (in mM) in experiments on DNA synthesis was the following: Tris-HCl (pH 8.3), 50; $MgCl_2$, 6; dithiotreitol, 4; KCl, 40; dATP, 0.1; dGTP, 0.1; dTTP, 0.1; [3H]dCTP (25Ci/mM), 0.02; a fragment of MS2 RNA, 5.5 $\mu g/ml$ or MS2 RNA, 60 $\mu g/ml$; octadeoxyribonucleotide or nonadeoxyribonucleotide (a primer), 2-5 $\mu g/ml$, reverse transcriptase, 20 units/ml, total volume 0.1 ml. After the incubation at 37°C for 1 hr, the product was deproteinized, precipitated with ethanol in the presence of tRNA-carrier, treated with 0.3 N KOH at 37°C for 17 hrs, neutralized, and passed through a column packed with Ultrogel AcA 34 and equilibrated with 0.05 M triethylammonium bicarbonate buffer (pH 6.7). The eluate containing labeled DNA was evaporated and the residue was dissolved in deionized water.

In experiments on the effect of the coat protein on synthesis of DNA on the MS2 RNA template, the RNA was preliminarily incubated with the coat protein under conditions of the complex formation or under the same conditions, but in the absence of the coat protein; then, a 10 μl aliquot of the solution was added to 0.1 ml of the incubation mixture which contained all components required for DNA synthesis, including a primer and reverse transcriptase. The mixture was incubated at 37°C; at definite periods of time, aliquots were taken and precipitated with TCA in the presence of the carrier RNA (1 mg/ml). The precipitates were then washed with 5 per cent TCA on nitrocellulose filters (AUFS, Chemapol), dried, and their radioactivity was counted in a toluene. The stability of a complex between the coat protein and MS2 [^{32}P]RNA at 37°C under conditions of DNA synthesis was measured by its binding to a nitrocellulose filter, but the mixture in this case did not contain a labeled deoxyribonucleoside triphosphate and reverse transcriptase. The lengths of the DNA products, synthe-

sized on the MS2 RNA template, were determined by electrophoresis in 98 per cent formamide ¹⁷ in plastic tubes (10x0.5 cm), the concentration of polyacrylamide gel being 4.5 per cent. After electrophoresis, gel slabs were scanned at 265 nm on a Scan 400 (Joyce Loebble) spectrophotometer and cut into fractions 1 mm thick. To determine their radioactivity, gel slices were placed into cuvettes containing 0.5 ml of a Protocol solution (NEN Company), kept at 50°C for 2 hrs, then cooled, and counted in 5 ml of toluene scintillation fluid by an Intertechnique SL 30 counter. The dimensions of the DNA product, synthesized on the MS2 RNA fragment, were determined by electrophoresis in polyacrylamide gel on slabs (1.5 x 29 x 0.04 cm) in 0.04 M Tris-citrate buffer (pH 8.0) ¹⁸ containing 6 M urea, the size of fractions being 1.5 x 0.5 cm.

RESULTS

The effectiveness of DNA synthesis in the presence of reverse transcriptase and an octadeoxyribonucleotide primer, which is complementary to the spacer between the cistrons of the coat protein and replicase, has been compared for several RNAs isolated from phages. As can be seen in Table 1, the effectiveness of transcription is almost identical for phages R17, MS2 and f2 that have similar non-translatable RNA regions between the cistrons of the coat protein and replicase ¹⁹⁻²¹. In contrast, RNA of a Q β phage, which has a different sequence in this region ²², is not transcribed in the presence of the given primer.

Table 1
Reverse transcription of various phage RNAs with
octanucleotide primer

Template RNA from phage	[³ H]dCMP, cpm/ μ g RNA
R17	1250
MS2	1550
f2	1500
Q β	120

The specific activity of [³H]dCTP in these experiments was 250 mC/mM; phage RNA, 60 μ g/ml.

Fig. 1. A. Structure of the genome of an MS2 phage and the intergenic non-translatable region.

B. Primary and secondary structures of the MS2 R(-53 → 6) fragment and a synthetic octadeoxynucleotide used as a primer-template in the reaction of reverse transcription.

The MS2 R(-53 → 6) fragment contains 59 nucleotides and comprises a region which is complementary to the octadeoxynucleotide primer; this fragment is protected with the coat protein from the hydrolytic action of T_1 RNase. The complex formation is highly specific because none of other MS2 RNA regions interacts with the coat protein¹³. That is way this RNA fragment can be isolated from a long polynucleotide chain after hydrolysis of nonprotected RNA of the RNA-protein complex. This MS2 R(-53 → 6) fragment whose structure is presented in Fig. 1B should form a complex with the primer and undergoes reverse transcription in the presence of reverse transcriptase.

Indeed, as can be seen in Fig. 2, intensive synthesis does take place; it depends entirely on the presence of a primer, and is partially inhibited by actinomycin D.

The cDNA made in these experiments was hybridized with the MS2 RNA fragment under conditions described earlier¹¹. The extent of hybridization was measured after S_1 -nuclease hydrolysis of the $[^3H]$ cDNA - MS2 R(-53 → 6) hybrid under conditions where double-helical regions are stable to hydrolysis. The 90% resistance was found compared to 8% resistance of the $[^3H]$ cDNA annealed without RNA fragment. This experiment proves the complementary nature of the synthesized cDNA with respect to MS2 RNA fragment used as a template.

It follows from Scheme B in Fig. 1 that the DNA product must be 52 nucleotides long because the primer is shifted by 7 nucleotides to the 3'-terminus of the fragment. Fig. 3A shows that the product is, in fact, of the length which should be expected in complete complementary copying of the RNA fragment. The absence of macromolecular fractions in the synthesized DNA (Fig. 3B) suggests that there is no slippage of the template in the given system.

As follows from these experiments, the octanucleotide primer may occupy the place which was postulated earlier⁵ for a system with an intact phage RNA. This does not mean however

Fig. 2. Incorporation of $[^3H]$ dCTP into the acid-insoluble fraction, catalysed by revertase, on the template of the MS2 R(-53 → 6) fragment:
 ▲ - in the absence of the primer;
 ● - in the presence of actinomycin D (100 μ g/ml);
 ○ - complete system without actinomycin D

that the primer will be bound at the same site of the entire phage RNA template, which is 60 times longer than the fragment, as in the case of the fragment where there is no choice. Taking into account the aforementioned ability of the coat protein to react specifically with this very region of RNA, we made an attempt to prove experimentally the specificity of the primer binding.

The experiment was conducted as follows. RNA of an MS2 phage was incubated with the coat protein under conditions which favoured the complex formation. Then, an aliquot of the solution was added to the reaction mixture which contained all the components required for DNA synthesis, including the primer and reverse transcriptase. In the control, MS2 RNA was preincubated under the same conditions, but in the absence of the coat protein.

Fig. 3. Polyacrylamide gel electrophoresis, under denaturation conditions, of a deoxypolynucleotide synthesized on the MS2 R(-53→6) template in the presence of a synthetic octadeoxy-nucleotide primer and revertase:

A. Separation in a system containing 12 per cent gel and 6 M urea.

1 - MS2 R(-53→50) fragment containing 103 nucleotides;
2 - tRNA (E.coli) detected in UV and containing 80 nucleotides;
3 - MS2 R(-53→6) fragment acting as a template in synthesis and containing 59 nucleotides; 4 - bromophenol blue.

As can be seen in Fig. 4, the coat protein inhibits the incorporation of a labeled precursor, and an incorporation into the acid-insoluble fraction is inversely proportional to the content of RNA in the complex.

The extent of binding is sufficient to account for the observed diminution of DNA synthesis. A sharp decrease of DNA synthesis in the presence of the coat protein occurs because this protein and the octanucleotide primer compete for binding to one and the same region of the RNA template; this region is no more accessible for the primer to be bound as soon as the coat protein has been preincubated with the MS2 RNA.

The slope of the curve (Fig. 4) describing the complex formation between coat protein and the MS2 RNA is due to slow thermal denaturation of the coat protein at 37° as was established in separate experiments (not shown).

It might be assumed that the primer non-specifically adsorbs on the coat protein; as a result, the effective concentration of the primer decreases, and reverse transcription is

Fig. 4. Incorporation of $[^3\text{H}]\text{dCTP}$ into the acid-insoluble fraction, catalysed by enzyme, on the MS2 RNA template: (o — o) in the presence of an octanucleotide primer and in the absence of the coat protein; MS2 RNA was preincubated under conditions used in the formation of a complex with the coat protein. (see Materials and Methods); (x — x) in the presence of an octanucleotide primer and the coat protein; MS2 RNA was preincubated under conditions of the formation of a complex with the coat protein; (● — ●) the same as (x — x), but in the presence of a nonanucleotide primer; (○ — ○) the amount of MS2 $[^{32}\text{P}]\text{RNA}$ in complex with the coat protein under conditions of DNA synthesis, determined by the binding of $[^{32}\text{P}]\text{RNA}$ with a nitrocellulose filter; (■ — ■) the same as (x — x) but in the presence of a nonanucleotide primer.

inhibited. This is not to be the case, as has been shown by experiments with another primer, a nonadeoxyribonucleotide which is complementary to the inner region of the coat protein cistron (Fig. 1A). This region of MS2 RNA does not interact with the coat protein, and DNA synthesis is not inhibited here (see Fig. 4).

Therefore, on the one hand, the primer can interact with the region of a spacer in the whole RNA molecule to which it is complementary; on the other hand, the interaction of this, and only this, RNA region with the coat protein sharply inhibits reverse transcription initiated by the octadeoxyribonucleotide primer, and the remaining activity is directly proportional to the content of a free phage RNA in a solution.

If the primer is located within the spacer region between the cistrons of the coat protein and replicase, cDNAs corresponding in length to the two cistrons of the coat pro-

Fig. 5. Polyacrylamide gel electrophoresis of $[^3\text{H}]$ DNA synthesized on the MS2 RNA template in the presence of an octanucleotide primer:

A. In the presence of actinomycin D (100 $\mu\text{g}/\text{ml}$); B. In the absence of actinomycin D. The markers used in the experiments were: 18S rRNA from ascite cells, 5S RNA from rat liver, and 4S RNA from yeast cells.

tein and A protein can be obtained in principle after complete complementary copying of MS2 RNA (Fig. 1A). That is why we have determined the length of the product being synthesized; to this end, DNA was analysed by electrophoresis in polyacrylamide gel under denaturation conditions (Fig. 5A). The product is heterogenous and consists of discrete peaks, the main peaks containing material 500 and 700 nucleotides long. Apart from these fractions which comprise the bulk of radioactivity, the product exhibits also small peaks corresponding to 1300 and 300 nucleotides in length.

The same fractions are formed in the absence of actinomycin D (Fig. 5B) as in its presence with an exception of two new peaks which are found in the region of short sequences. Though their nature has not been investigated, it might be presumed that the appearance of these peaks is related to synthesis of an anticomplementary DNA strand.

DISCUSSION

In this and the previous ⁵ papers, it has been shown for the first time that short synthetic heteropolymeric deoxyribonucleotides can act as primers in reverse transcription of RNA templates. This approach has been used earlier in DNA synthesis on a DNA template, catalysed by DNA-polymerase I ²⁴. In this case, the (+) chain of DNA of a f1 phage was used as a template, and a synthetic octadeoxyribonucleotide, as a primer. As a result, a unique sequence was synthesized, i.e. the initiation started at a definite, topographically fixed, point.

Reverse transcription can be initiated, as is shown in this work, either from the intercistron region or from a sequence within the structural gene. In both cases, the primer is not located at the 3'-terminus of the template, but is complementary to the inner region of an RNA chain, similar to a natural primer of oncornaviral RNAs, tRNA. Synthesis with one and the same primer is more effective when a fragment of a phage RNA is used rather than the whole phage RNA. This may be due to the fact that a region, which is complementary to the primer, is more exposed to intermolecular interactions in the fragment than in the whole RNA molecule.

The possibility to obtain complete transcripts of various macromolecular RNAs ^{3,4,25-27}, and elements of the secondary structure found in cDNAs ^{28,29} suggest that the secondary structure of a template is not, in principle, an obstacle to reverse transcription. The data on complete complementary copying of the RNA fragment consisting of two hairpins can serve as direct proof (Fig. 1B).

According to the data obtained with the fragment of R17 RNA ³⁰, the melting point of hairpin (1) (Fig. 1B), under conditions similar to those of incubation for DNA synthesis, is 88°C whereas T_m of hairpin (2) is 64°C. Even if T_m of hairpin (1) decreases due to a lower concentration of Mg^{2+} and substitution of an AC pair for an AU pair, when the fragment of MS2 RNA is used instead of the fragment of R17 RNA, T_m of hairpin should not be below 70°C, this being by 30°C higher than the temperature at which the synthesis was conducted. Hairpin (2) apparently melts to a considerable extent because its intramolecular stability decreases as soon as a complex with the oligonucleotide primer has been formed, and this makes the paired region shorter by at least two GC pairs (see Fig. 1A).

As has been shown separately (unpublished) the efficiency of complex formation between MS2 RNA fragment and the octanucleotide at the given temperature and concentrations of the reacting molecules does not exceed 10-15%. In other words, only a limited fraction of the RNA templates do contain hydrogen-bonded primer. The extent of the cDNA synthesis was calculated to correspond to 6-8% of the total number of templates present in the incubation mixture. From comparison of these two values it follows that about half of all the oligonucleotide molecules attached to the template was utilized as primers. Therefore, the synthesis could not involve only a negligible fraction of the template molecules whose secondary structure might be lost as a result of thermal fluctuations ³⁰. The length of the cDNA product proves that hairpin (1) has been completely transcribed. Consequently, the secondary structure of the RNA template does not hinder reverse transcription under the action of reverse transcriptase.

It does not follow however that the secondary structure of the template is of no importance to the action of the enzyme. An increase in temperature and a decrease in ionic strength are known to elongate DNA chains being synthesized²⁶; moreover, "structural" stops are observed in synthesis, and these stops are probably related to the influence of the template secondary structure^{25,3}. "Structural" stops are also responsible apparently for the discrete character of synthesized DNA fractions (Fig. 5). Distances from the initiation point for transcripts of different length correspond approximately to a region in the cistron of the coat protein between two large hairpins, the end of the cistron of the A protein, and inner regions in the cistron of the A protein. The length of a complete transcript must be ~1750 nucleotides. The region corresponding to this length is radioactive after gel electrophoresis (Fig. 5), which suggests that a small fraction of complete transcripts is present in the preparation.

The "melting" ability of revertase seems to be enough for the complementary copying of oncornaviral and probably a number of other RNAs in vivo. However, it is probably not sufficient (under conditions similar to those in vivo) to use highly structured RNAs as templates of reverse transcription.

The hypothesis of the "unwinding" action of revertase provides explanation for findings that oligomerization of the enzyme is required if synthesis occurs on a template with the secondary structure, and is not necessary when a template does not have the secondary structure³¹. In the former case, a molecule of revertase binds to the template and catalyses the reaction; another, or other, molecule(s) of the enzyme, which are associated with the first molecule, unwind the template, thus preparing it for the synthesis. Synthetic templates that lack hairpins do not require "unwinding" molecules, and the synthesis can be catalysed by the monomeric form of revertase.

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REFERENCES

- 1 Temin, H.M. and Mizutani, S. (1970) *Nature* 226, 1211-1213.
- 2 Baltimore, D. (1970) *Nature* 226, 1209-1211.
- 3 Haseltine, W.A., Kleid, D.G., Panet, A., Rothenberg, E. and Baltimore, D. (1976) *J. Mol. Biol.* 106, 109-131.
- 4 Kacian, D.L. and Myers, J.C. (1976) *Proc. Nat. Acad. Sci. USA* 73, 2191-2195.
- 5 Metelev, V.G., Stepanova, O.B., Smirnov, V.D., Shabarova, Z.A., Prokofyev, M.A., Frolova, L.Yu., Graevskaya, N.A., and Kisselev, L.L. (1974) *Dokl. Akad. Nauk SSSR* 214, 957-960.
- 6 Kisselev, L.L., Haenni, A.-L. and Chapeville, F. (1976) *FEBS Letts.* 62, 64-68.
- 7 Green, M. and Gerard, G.F. (1974) *Progr. Nucl. Acid. Res. and Mol. Biol.* 14, 188-334.
- 8 Frolova, L.Yu. (1976) *Molecular Biology (series) v. 7*, p. 142-193, VINITI, Moscow.
- 9 Getz, M.J., Birnie, G.D. and Paul, J. (1974) *Biochemistry* 13, 2235-2240.
- 10 Kacian, D.L., Watson, K.F., Burny, A. and Spiegelman, S. (1971) *Biochem. Biophys. Acta* 246, 365-383.
- 11 Kisselev, L.L., Frolova, L.Yu., Gazarian, K.G., Tarantul, V.Z. and Engelgardt, V.A. (1973) *Dokl. Akad. Nauk SSSR* 213, 1203-1206.
- 12 Berzin, V., Rosenthal, G. and Gren, E.J. (1974) *Eur. J. Biochem.* 45, 233-242.
- 13 Berzin, V.M., Borisova, G.P., Gribov, V.A., Rosenthal, G.F., Tsielens, I.E., Yansone, I.V. and Gren, E.J. (1976) *Dokl. Akad. Nauk SSSR* 229, 741-744.
- 14 Sugiyama, T., Herbert, R.R. and Hartman, K.A. (1967) *J. Mol. Biol.* 25, 455-463.
- 15 Weber, H. and Khorana, H.G. (1972) *J. Mol. Biol.* 72, 219-249.
- 16 Smirnov, V.D., Ratmanova, K.I., Shabarova, Z.A. and Prokofyev, M.A. (1975) *Dokl. Akad. Nauk SSSR* 223, 121-123.
- 17 Pinder, J.C., Staynov, D.Z. and Gratzner, W.B. (1974) *Biochemistry* 13, 5373-5377.
- 18 De Wachter, R. and Fiers, W. (1972) *Anal. Biochem.* 49, 184-197.
- 19 Bernardi, A. and Spahr, P.F. (1972) *Proc. Nat. Acad. Sci. USA* 69, 3033-3037.
- 20 Nichols, J.L. and Robertson, H.D. (1971) *Biochim. Biophys. Acta* 228, 676-681.
- 21 Min, Jou W., Haegeman, G. and Fiers, W. (1971) *FEBS Letts.* 13, 105-109.
- 22 Weber, H. (1976) *Biochim. Biophys. Acta* 418, 175-183.
- 23 Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min, Jou W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Vokckaert, G. and Ysebaert, M. (1976) *Nature* 260, 500-507.
- 24 Sanger, F., Donelson, J.E., Coulson, A.R., Kössel, H. and Fischer, D. (1973) *Proc. Nat. Acad. Sci. USA* 70, 1209-1213.
- 25 Efstratiadis, A., Maniatis, T., Kafatos, F.C., Jeffrey, A. and Vournakis, J.N. (1975) *Cell* 4, 367-378.

- 26 Monahan, J.J., Harris, S.E., Woo, S.L.C., Roberson, D.L. and O'Malley, B.W. (1976) *Biochemistry* 15, 223-233.
- 27 Junghans, R.P., Duesberg, P.H. and Knight, C.A. (1975) *Proc. Nat. Acad. Sci. USA* 72, 4895-4899.
- 28 Gasaryan, K.G., Tarantul, V.Z., Baranov, Yu.N., Frolova, L.Yu. and Kisselev, L.L. (1974) *Mol. Biol. Rep.* 1, 465-470.
- 29 Proudfoot, N.J. and Brownlee, G.G. (1974) *Nature* 252, 359-362.
- 30 Gralla, J., Steitz, J.A. and Crothers, D.M. (1974) *Nature* 248, 204-208.
- 31 Yamaura, I. and Cavalieri, L. (1976) *J. Virol.* 18, 26-33.