Ribosomal frameshifting at the *Gag-Pol* junction in avian leukemia sarcoma virus forms a novel cleavage site

G. Arad, R. Bar-Meir, Moshe Kotler*

Department of Molecular Genetics, The Hebrew University, Hadassah Medical School, POB 1172, Jerusalem 91010, Israel

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Abstract The *Gag* and *Gag-Pol* precursors of avian sarcoma leukemia virus (ASLV) are translated from viral genomic-size mRNA at a molar ratio of about 20:1. Translation of *Gag* is terminated at the stop codon UAG located at the carboxyl-terminus of the viral protease (PR), whereas a ribosomal frameshift occurring at the carboxyl-terminus of *Gag* allows translation of the *Gag-Pol* precursor. To determine how PR is released from the *Gag-Pol* precursor, a single base (A or T) was inserted at the *Gag-Pol* junction in order to adjust the translation into a single reading frame. These mutations allow processing of the viral precursor when expressed in bacterial cells, but cause cessation of viral production after transfection of avian cells. The viral PR released from the large precursor is one amino acid longer than PR cleaved from the *Gag* polyprotein and is terminated by an Ile instead of a Leu residue.

Key words: Protease; Frameshift; *Gag-Pol*; Fusion protein; Processing

1. Introduction

Retroviral *Gag* and *Gag-Pol* precursors are translated from the genomic-size mRNA [1]. In avian sarcoma leukemia viruses (ASLV) the *Gag-Pol* precursor is translated via a ribosomal frameshift (FS) which takes place at the carboxyl-terminus of *Gag* [2,3]. The *Gag* and *Gag-Pol* precursors are cleaved into mature structural and enzymatic proteins by a viral-encoded protease (PR) during virion assembly or even after virus release. The 15 kDa (124 amino acid residues) PR of ASLV resides at the carboxyl-terminus of $pr76^{Gag}$ and at the *Gag-Pol* junction of $pr180^{Gag\text{-}Pol}$ precursors, while in mammalian retroviruses it is a part of the *Gag-Pol* precursor only. The production of $pr76^{Gag}$ and $pr180^{Gag\text{-}Pol}$ polyproteins at a molar ratio of 20:1 is regulated by ribosomal frameshifting which takes place at the A AAT TTA TA region located at the 3′ end of the *gag* region, and which codes for Asn-Leu-Ile at the *Gag-Pol* junction [1–3].

The HIV-1 *Gag* and *Pol* genes positioned in the same translational reading frame underwent autocatalysis when expressed in COS cells [4]. On the other hand, ASLV *Gag-Pol* fusion polyprotein expressed in avian and mammalian cells remained unprocessed. The processing of this viral $pr180^{Gag\text{-}Pol}$ can be complemented by co-expression of the *Gag* protein from a separate vector [5]. To determine whether ASLV protease embedded in the *Gag-Pol* precursor is enzymatically active, we expressed the fusion proteins in bacterial cells and followed their autoprocessing.

Here we demonstrate that the ribosomal frameshift which is essential for virus production, generates a novel cleavage site for viral PR. *Gag* and *Pol* polyproteins which were adjusted to a single reading frame undergo autocleavage in bacterial cells to release PR and mature viral proteins. Cleavage by viral PR of synthetic peptides homologous to the *Gag-Pol* junction suggests that PR released from the $pr180^{Gag\text{-}Pol}$ precursor contains 125 amino acid residues instead of the 124 present in PR cleaved from $pr76^{gag}$, and that Ile replaces Leu at the carboxyl-terminus.

2. Materials and methods

2.1. Bacterial cells

The *E. coli* strain MC1061 was used as the host for all expression vectors. The cells contained the plasmid pRK248cIts, which directs the expression of a temperature-sensitive bacteriophage lambda repressor protein [6].

The *E. coli* strain DR100 was used as the host for pBR322 containing the permutated Schmidt Ruppin A provirus (pSRA) [7] obtained from Dr. S. Hughes (National Cancer Institute, Frederick, MD). This vector was used for preparing the FS- mutated proviral DNAs for the transfection experiments.

2.2. Construction of bacterial clones

A viral *Pst*I–*Bam*HI fragment (2455–3708 [8]) derived from pATV8, a clone of the avian sarcoma Prague C strain [9], was inserted into the pUC18 plasmid. This construct was used to mutate the *gag-pol* frameshift region by site-directed mutagenesis, as described before [10,11]. The oligonucleotides used for mutagenesis were derived from the wt sequence:

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wt- 5′CGC TTA ACA AAT TTA TAG GGA GGG CCA CTG TTC TCA
FS1- 5′CGC TTC ACA AAT TTAaTA GGt cGa GCC ACT GTT CTC
FS2- 5′CGC TTG ACA AATtTT ATA GGT cGa GCG ACT GTT CTC
FS3- 5′CGC TTG ACA AATtTT ccc GGG AGA GCC ACT GTT CTC
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(small letters indicate the mutated bases).

The wild type (wt) and the mutated *Pst*I–*Bam*HI fragments were excised from the pUC18 constructs and ligated to a *Bam*HI–*Pst*I fragment derived from pRT [12] and to the *Pst*I–*Pst*I fragment taken from pPR, pNC-PR or $pNC\text{-}PR^{Asp^{37}Ile}$ [13] to generate plasmids with pFS1 and pFS2. The *Pst*I–*Pst*I fragment supplies the lambda phage promoter operator region (P_LO_L), a ribosomal binding site and translation initiator codon in frame with the viral CA protein. The *Pst*I–*Pst*I fragment derived from $pNC\text{-}PR^{Asp^{37}Ile}$ provides an inactive PR [10]. Expression of the viral proteins in bacterial cells was induced by shifting cultures from 30°C to 42°C for 3–6 h, and virus-related proteins were detected by immunoblotting as described elsewhere [10].

Plasmids containing the viral genomic DNA with the frameshift mutations (pSRAFS1-3) were generated by ligation of *Sac*I–*Eco*RI and *Sac*I–*Kpn*I fragments of pSRA with the *Eco*RI–*Kpn*I fragments derived from pFS1, 2, or 3.

2.3. Transfection experiments

DNAs prepared from pSRAFS1, 2 and 3 were linearized by cutting with *Sal*I and ligated to form linear genomic viral molecules. Subconfluent QT-6 cells [14] were transfected with 10 μg of wt or mutated proviral DNA per 100 mm culture plate (Nunc), using a standard calcium phosphate transfection protocol [15].

*Corresponding author.

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2.4. Reverse transcriptase assay

Media harvested from transfected QT-6 cultures were cleared of cell debris by centrifugation. 5 μl of the supernatant was used in 10 μl reaction medium to detect reverse transcriptase activity using poly-A:oligo-dT as primer template and [^{32}P]TTP (specific activity 3000 mCi/mmol; New England), as described before [16].

2.5. Synthesis and cleavage of peptide substrates

The synthetic peptides:

FS1- NH_2 Arg Leu Thr Asn Leu Ile Gly Arg Ala Thr COOH
FS2- NH_2 Arg Leu Thr Asn Phe Ile Gly Arg Ala Thr COOH
FS3- NH_2 Arg Leu Thr Asn Phe Pro Gly Arg Ala Thr COOH

have the same amino acid sequence as the mutated *Gag-Pol* junctions. Decapeptide NH_2 Thr Phe Glu Ala Phe–Pro Leu Arg Glu Ala COOH is homologous to the ASLV RT–IN junction and NH_2 Thr Phe Glu Ala Ala–Pro Leu Arg Glu Ala COOH contains a mutated amino acid which prevents cleavage by the viral PR. All synthetic peptides were synthesized by the solid-phase method [17].

Proteolytic reactions were carried out in 20 μl of 0.1 M sodium citrate buffer (pH 5.5) containing 16 pmol of PR, 7.7 nmol of a decapeptide substrate and 0.4 M sodium chloride, at 37°C for 1 h. Reactions were stopped by incubation at 100°C, and the reaction products were analyzed by HPLC as described before [18].

2.6. Amino acid analysis

Amino acid content was determined by the PICOTAG method on a Water Chromatography Division with minor modifications.

3. Results

3.1. Ribosomal frameshift is essential for viral production

Plasmids containing the SRA and the mutated SRA viral DNA (pSRAwt, pSRAFS1, pSRAFS2 and pSRAFS3) were used to transfect QT-6 cell cultures in order to verify that the ribosomal frameshift is indeed essential for ASLV production in permissive cells. Media collected from the transfected cells were clarified by centrifugation and assayed for reverse transcriptase (RT) activity. As can be seen in Fig. 1, particles with RT activity were detected in media harvested from cultures transfected with pSRAwt, but not in media collected from those transfected with viral DNA containing the *gag–pol* region in frame (pSRAFS1, -2 and -3). In addition, media harvested from cells transfected with pSRAwt, but not with pSRAFS1 and pSRAFS2, contained viral particles which were detected with anti-CA sera and which were infectious in fresh QT-6 cells (data not shown). Similar results, demonstrating that the production of ASLV and HIV-1 particles are dependent on ribosomal frameshifting and that murine leukemia virus production is dependent on the amber codon located between the *gag–pol* regions, have been published previously [4,5,19–21]. It is possible that PR expressed as part of pr180$^{Gag\text{-}Pol}$ is not active in processing viral precursors.

3.2. Processing of ASLV Gag-Pol *fusion proteins expressed in* E. coli

Gag-Pol polyproteins containing the NC-PR-RT-IN fragment with Leu-Ile-Gly (FS1) or Phe-Ile-Gly (FS2) at the *Gag-Pol* junctions were expressed in bacterial cells. Immunoblots of bacterial extracts, when developed with anti-PR (Fig. 2A), showed that both polyproteins underwent autocleavage to release PR. The PR released from the *Gag-Pol* polyproteins had a migration coefficient similar to PR expressed alone or released from NC-PR dipeptides [10]. PR was not released when mutated inactive PR$^{Asp^{37}Ile}$ was expressed as part of pFS1 and

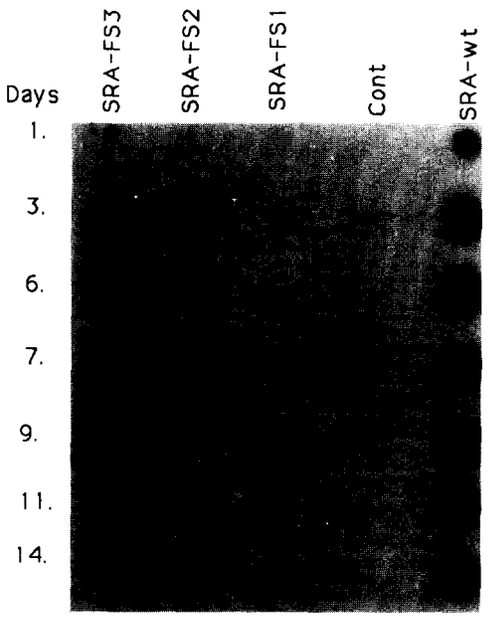

Fig. 1. Reverse transcriptase assay of transfected QT-6 cell culture supernatant. QT-6 cultures were transfected with pSRAwt and pSRAFS clones. 24, 48 and 72 h post-transfection, the reverse transcriptase level was determined in the culture supernatant. ^{32}P-Labeled DNA products were spotted onto DEAE paper as previously described [16], and the paper was exposed to X-ray film. Media from untransfected cells and from pSRA-transfected cells were used as negative and positive controls.

pFS2, indicating that processing of the viral precursors was carried out by viral, and not cellular, proteins. Several proteins, larger than viral PR and reactive to anti-PR, were present on the blot (Fig. 2A). These proteins resulted from partial processing of the polyproteins.

Fig. 2B and C demonstrate that the NC-PR-RT-IN fusion proteins containing FS1 or FS2 sequences underwent processing in bacterial cells. The viral proteins are cleaved into *Pol* precursor pr95 and into the mature proteins p63 (RT) and p32 (IN). Thus the pr95 reacts with anti-RT and anti-IN (Fig. 2B and C) while the p32 and p63 proteins are detected in B and C, respectively. The pPR$_{FS1}$RT-IN is cleaved into IN (p32) and RT (p63) but not into pr95 (B and C).

3.3. Cleavage of synthetic peptides representing the Gag-Pol *junction*

The synthetic peptides FS1 and FS2 were used as substrates for PR hydrolysis in order to determine the cleavage sites at the ASLV *Gag-Pol* junction. Fig. 3 clearly indicates that decapeptides FS1, but not FS2, are cleaved by PR, implying that a decapeptide containing Asn Leu Ile Gly, but not Asn Phe Ile Gly, is an efficient substrate for PR (Fig. 3). Decapeptides containing Phe-Pro or Ala-Pro at the cleavage site were used as positive and negative controls, respectively, for the PR activity as shown before [18].

Amino acid analysis of the cleavage products isolated after PR hydrolysis showed that product #1 contained Arg, Leu, Thr, Asn, Ile; and product #2 contained Gly, Arg, Ala, Thr. These results indicate that the FS1 decapeptide was cleaved between the Ile and Gly residues. If in vitro cleavage of the

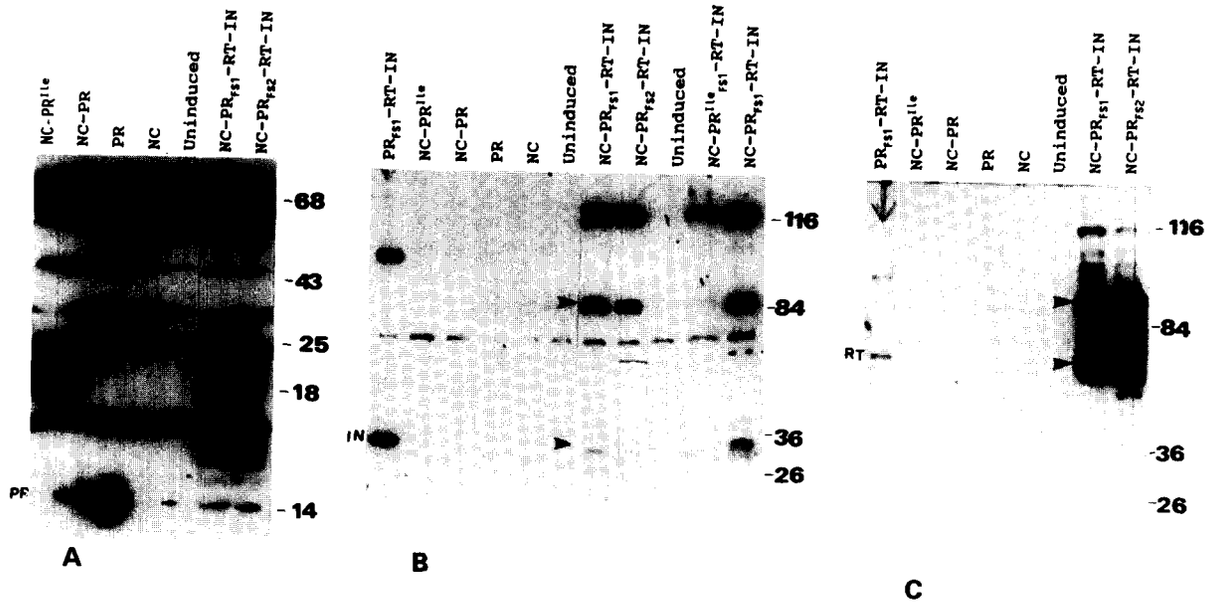

Fig. 2. Expression and processing of ASLV polyproteins in *E. coli*. Lysates of *E. coli* strains that express the indicated viral polyproteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose paper. ASLV polypeptides were detected by antibody to PR (A), anti-IN (B) and with anti-RT (C). Reaction products were detected with ^{135}I-labeled protein A. Molecular weights (×1000) are indicated on the left. The arrows indicate the position PR, IN and RT released from the viral polyproteins.

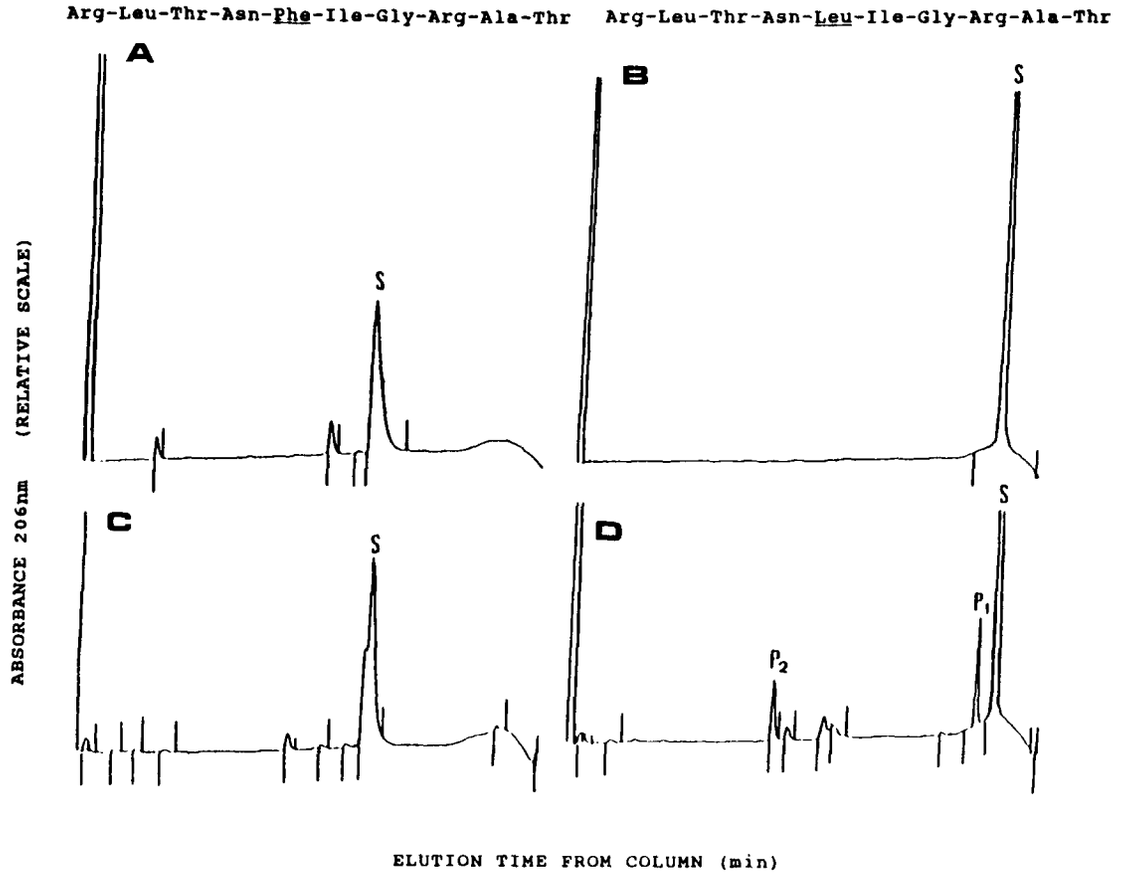

Fig. 3. Comparison of cleavage products of FS1 and FS2 peptides by using purified ASLV PRs. Reaction mixtures containing FS2 (A and C), or FS1 peptides (B and D) were incubated at 37°C with (lower panels) or without (upper panels) purified PR. Samples were taken after 60 min incubation time and were analyzed by HPLC. S, P1 and P2 indicate substrate and cleavage products, respectively.

synthetic peptides indeed mimics the in vivo processing of the viral *Gag-Pol* precursor, then PR released from pr180$^{gag\text{-}pol}$ contains 125 amino acid residues instead of the 124 in PR released from the pr76^{gag} precursor. Ile is the amino acid at the carboxyl-terminus of PR released from pr180$^{Gag\text{-}Pol}$, being the 125th residue, as opposed to #124, Leu, positioned at the carboxyl-terminus of *Gag*.

4. Discussion

Mature avian sarcoma leukemia virions contain free PR molecules which are probably released from the pr76^{gag} and pr180$^{gag\text{-}pol}$ precursors. Release of PR and other viral proteins from *Gag-Pol* polyproteins expressed in bacterial cells via FS1 and FS2 mutations suggests that the PR embedded in the *Gag-Pol* precursor is enzymatically active and that amino acid sequences located at the carboxyl region of PR contain a cleavage site for the viral PR. Previously, it was shown that the ASLV but not HIV-1 *Gag-Pol* fusion proteins expressed in mammalian cells was unable to carry out auto-processing [4,5]. It is therefore possible that correct conformation of the PR domain in the fusion protein or/and high local concentrations of the large precursors are required to activate the PR embedded in the *Gag-Pol* precursors.

Decapeptides containing the junction region of mature *Gag-Pol* proteins are specific and efficient substrates for viral PR [18,22,23]. Because it is extremely difficult to analyze the carboxyl-terminus of PR released from pr180$^{Gag\text{-}Pol}$, we assayed whether FS1 and FS2 decapeptides are cleaved by purified ASLV PR. Based on the amino acid composition of the cleavage products, we suggest that PR released from the *Gag-Pol* precursor contains 125 amino acid residues, instead of the 124 residues in PR released from the *Gag* precursor. In contrast to the results obtained in bacterial cells, where PR was released from polyproteins expressed via FS1 and FS2 mutations, synthetic decapeptides homologous to FS1 were cleaved by PR in vitro. However, it is possible that in bacterial cells, PR is released from the FS2 frameshift polyprotein by cleavage at a site which is not accessible for PR hydrolysis in the FS2 decapeptide.

Jacks and co-workers [2,3] demonstrated that a ribosomal frameshift occurring during translation of ASLV genomic mRNA leads to expression of Leu-Ile residues (FS1) at the *Gag-Pol* junction. However, an additional A to U 'slippery site' resulting in a Phe-Ile junction (FS2) may exist, as suggested by Majors [24]. At present, we cannot negate the possibility that the *Gag-Pol* precursor is formed via FS1 and FS2. However, translation via FS1 is a more favorable possibility since translation of a sequence coding for Leu-Ile was demonstrated in vitro [2,3], and this junction creates a cleavage site very close to the terminus of PR released from the *Gag* precursor.

It is of interest that retroviral proteases cleave the precursors close to the *Gag-Pol* junction. For example, in HIV-1, as in ASLV, PR cleaves 6–7 amino acids downstream of the frameshift sites, namely at the amino-termini of RT and p6^{Pol} of ASLV and HIV-1, respectively [25–27]. Additional cleavage sites are present close to the frameshift site, namely in ASLV Ile/Gly at the *Gag-Pol* junction (as shown in this report), and in HIV-1 Asn/Phe at the NC/P1 junction [28]. It therefore seems that over evolutionary times a mechanism has been preserved which ensures complete separation of the translation products of *Gag* and *Pol*.

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References

[1] Coffin, J.M. (1990) In: Virology: Retroviridae and their replication, vol. 2 (Fields, B.N. and Knipe, D.M. Eds.) pp. 1437–1500, Raven Press, New York.
[2] Jacks, T., Madhani, H.D., Masiarz, F.R. and Varmus, H.E. (1988) Cell 329, 447–458.
[3] Jacks, T. and Varmus, H.E. (1985) Science 230, 1237–1242.
[4] Park, J. and Morrow, C.D. (1991) J. Virol. 65, 5111–5117.
[5] Craven, R.C., Bennett, R.P. and Wills, J.W. (1991) J. Virol. 65, 6205–6217.
[6] Crowl, R., Seamans, C., Lomedico, P. and McAndrew, S. (1985) Gene 38, 31–38.
[7] Hughes, S. and Kosik, E. (1984) Virology 136, 89–99.
[8] Schwartz, D.E., Tizard, R. and Gilbert, W. (1983) Cell 32, 853–869.
[9] Katz, R.A., Omer, C.A., Weiss, J.H., Mitsiallis, S.A., Faras, A.J. and Guntaka, R. (1985) J. Virol. 42, 346–351.
[10] Kotler, M., Katz, R.A. and Skalka, A.M. (1988) J. Virol. 62, 2696–2700.
[11] Morinaga, Y., Franceschini, T., Inouye, S. and Inouye, M. (1984) BioTechnol. 2, 636–639.
[12] Alexander, F., Leis, J., Soltis, D.A., Crowl, R.M., Danho, W., Poonian, M.S., Pan, Y.-C. and Skalka, A.M. (1987) J. Virol. 61, 534–542.
[13] Burstein, H., Bizub, D., Kotler, M., Schatz, G., Vogt, V.M. and Skalka, A.M. (1992) J. Virol. 66, 1781–1785.
[14] Moscovici, C., Moscovici, M., Jimenez, H., Lai, M.M.C., Hayman, M.J. and Vogt, P.K. (1977) Cell 11, 95–103.
[15] Demetrios, A.S. and Welkie, N.M. (1984) in: Transcription and Translation: Expression of exogenous DNA in mammalian cells (Hames, B.D. and Higgins, S.J. Eds.) pp. 1–45, IRL Press, Boca Raton, FL.
[16] Goff, S., Traktman, P. and Baltimore, D. (1981) J. Virol. 38, 239–248.
[17] Tam, P.J., Health, W.F. and Marrifield, R.B. (1983) J. Am. Chem. Soc. 105, 6442–6455.
[18] Kotler, M., Katz, R.A., Danho, W., Leis, J. and Skalka, A.M. (1988) Proc. Natl. Acad. Sci. USA 85, 4185–4189.
[19] Oertle, S., Bowles, N. and Spahr, P.F. (1992) J. Virol. 66, 3873–3878.
[20] Karacostas, V., Wolffe, E.J., Nagashima, K., Gonda, M.A. and Moss, B. (1993) Virology 193, 661–671.
[21] Felsenstein, K.M. and Goff, S.P. (1988) J. Virol. 62, 2179–2182.
[22] Tomasseli, A.G., Howe, W.J., Sawyer, T.K. and Wlodawer, A. (1991) Int. J. Biochem. Biotech. 4, 7–27.
[23] Oroszlan, S. and Luftig, R.B. (1990) Curr. Top. Microbiol. 157, 153–185.
[24] Majors, J. (1990) Enzyme 44, 320–331.
[25] Phylip, L.H., Mills, J.S., Parten, B.F., Dunn, B.M. and Kay, J. (1992) FEBS Lett. 314, 449–454.
[26] Zybarth, G., Krausslich, H.-G., Partin, K. and Carter, C. (1994) J. Virol. 68, 240–250.
[27] Arad, G., Almog, N., Passi-Even, L., Wainberg, M.A. and Kotler, M. (1995) in preparation.
[28] Henderson, L.E., Bowers, M.A., Sowder, R.C., Serabyn, S.A., Johnson, D.G., Bess, J.W., Arthur, L.O., Bryant, D.K. and Fenselau, C. (1992) J. Virol. 66, 1856–1865.