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Covalent chromatography of influenza virus membrane M1 protein on activated thiopropyl Sepharose-6B

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Abstract

The M1 protein of influenza virus is a highly hydrophobic polypeptide that is resistant to enzyme cleavage during incubation in water solutions. We show here that the M1 protein that is immobilized on an insoluble activated support (thiopropyl Sepharose-6B) by means of a thiol–disulfide exchange reaction acquires sensitivity to trypsin. After tryptic digestion noncysteine-containing peptides of M1 were removed by washing the support, while cysteine-containing ones were detached from the support by reduction. As a result, 24 unique tryptic peptides of M1 protein were clearly separated by reversed-phase high-performance liquid chromatography. The described method opens a new way to the investigation of functional properties of distinct domains of viral thiol proteins. © 1998 Elsevier Science B.V.

Keywords: Influenza virus; Matrix M1 protein; Tryptic peptides

1. Introduction

Influenza virus is a typical enveloped virus [1,2]. The most abundant matrix M1 protein is localized between the lipoprotein envelope and the ribonucleoprotein (RNP) complex and is believed to bridge these subviral components thereby maintaining the structural integrity of the viral particle (see review Ref. [3]). This virion structure suggests an important role for the M1 protein both in the stage of fusion between viral and endosomal membranes and in the stage of intracellular transport and of viral RNP functioning. To understand M1 protein function it is necessary to study the functional properties of its distinct domains (such as interaction with host proteins, with viral RNP and with different antibodies of influenza infected animals and humans).

Until now, such studies were limited because M1 protein is highly hydrophobic and poorly soluble in water. Consequently, it can bind nonspecifically to various membranous components during attempted isolation. We show that the M1–RNP linkages in virion are sensitive to acidic pH and that solubilization of the M1 protein depends both the salt and hydrogen ion concentration [4]. We extended these findings by developing a simple method to isolate the soluble monomeric M1 protein [5] and we applied the method to begin the study of the M1 protein interaction with cellular polypeptides [6].

Recently, we found that such solubilized M1 protein remains resistant to enzyme cleavage during incubation. To overcome this problem a combined covalent chromatography method with activated

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thiopropyl Sepharose-6B as a support has been used [7]. In this method purified M1 was immobilized on thiopropyl Sepharose-6B via the thiol–disulphide reaction followed by trypsin digestion and chromatographic isolation of generated peptides. As a result 24 peptides of M1 protein were isolated by this method and characterized.

2. Experimental

2.1. Reagents and chemicals

Thiopropyl activated Sepharose-6B was obtained from Pharmacia (Uppsala, Sweden); trypsin treated with L-1-tosylamide-2-phenylchloromethylketone (TPCK–trypsin) and grade 1 guanidine–hydrochloride (Gu–HCl) were obtained from Sigma (St. Louis, MO, USA). β-Mercaptoethanol (β-ME) and diisopropylfluorophosphate (DFP) were from Merck (Darmstadt, Germany). Water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were analytical grade.

2.2. Isolation and purification of M1 protein

Influenza virus A/Aichi/2/68 (H3N2) was propagated on the allantoic fluid of embryonated chicken eggs [8]. The method of M1 purification was described in detail earlier [5]. Briefly, virus-containing allantoic fluid was clarified by centrifugation (10 000 *g*, 30 min), loaded over 20% glycerol containing 100 m*M* NaCl, 2 m*M* Tris–HCl (pH 7.8) and 2% nonionic detergent, Nonidet P-40 (NP-40) and centrifuged at 50 000 *g* at 12°C for 2.5 h (SW·27 rotor; Spinco L7) to pellet viral core structures. Pellets were resuspended in 0.4 ml 50 m*M* 2-[N-morpholino]ethansulfonic acid (pH 3.5), containing 5% glycerol, 100 m*M* NaCl and 0.1% NP-40 and in addition centrifuged at 10 000 *g* for 10 min in a table centrifuge. Supernatant, which contain soluble M1 protein was removed for further examination. Using this method we succeeded in obtaining about 250 μg (10 nmol) protein in 450–500 μl from 1 ml of viral suspension at a concentration of 1 mg·ml^{-1}. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis analysis of the M1 protein was carried out according to Laemmli [9] using 12% polyacrylamide gel slabs. M1 protein solution 2–3 μml was diluted with 5 μl 0.15 *M* Tris–HCl buffer (pH 6.8) containing 20% glycerol, 6 *M* urea, 2% SDS and 0.01 *M* dithiotrietol and heated for 1 min in boiling water before electrophoresis.

2.3. Tryptic digestion of M1 protein

M1 protein was alkylated with 4-vinylpyridine after reduction of disulfide-bonds as described [10]. Protein samples were then desalted by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Nucleosil 300 C_4 75×4.6 mm I.D. column (Elsico, Moscow, Russia). M1 protein was collected and concentrated using a SpeedVac centrifugal evaporator. Ammonium bicarbonate buffer (1 *M*, pH 7.8) was added followed by addition of TPCK–trypsin (enzyme:substrate ratios were 1:100 and 1:50, w/w). The reaction was carried out at 37°C for 4 h at constant stirring. Aliquots of protein, 1–5 μg, were removed before and after the reaction to evaluate the degree of hydrolysis. Reactions were stopped by the addition of 2 μg inhibitor (DFP).

2.4. HPLC equipment and conditions

A Beckman 344 chromatograph (Beckman, Berkeley, CA, USA) with a 165 UV–Vis dual channel spectrophotometric detector was used at 215 and 280 nm. Columns with Nucleosil 300 C_4 (Macherey–Nagel, Düren, Germany), 75×4.6 mm I.D. (Elsico, Moscow, Russia), and with Ultrasphere ODS column 250×4.6 mm I.D. from Beckman were used. The samples were dissolved in the starting eluent. The eluent flow was 1.0 ml·min^{-1} in each case. All separations were performed at room temperature (20±3°C). Protein samples were separated on a Nucleosil 300 C_4 column with linear gradient (0–60% at 30 min) of acetonitrile in water with 0.1% trifluoroacetic acid (TFA). The noncysteine-containing and cysteine-containing tryptic peptides were separated on an Ultrasphere ODS column using 0–60% in 60 min and 60–80% in a 10 min linear gradient of acetonitrile in water with 0.1% TFA. Peptide yields were 30%. Fractions were collected

using a Gilson 201 fraction collector for subsequent analysis.

2.5. Analytical methods

The tryptic peptides were hydrolysed as described by Tsugita and Scheffler [11]. Amino acid analysis was made on a Hitachi-835 analyser (Tokyo, Japan) in the standard mode for protein hydrolysate analysis with cation-exchange separation and ninhydrin post-column derivatization.

The short N-terminal amino acid sequences of the tryptic peptides were determined on a model 810 gas-phase protein sequencer (Knauer, Berlin, Germany) equipped with a 120A PTH-analyser and a 140A separation system (Applied Biosystems, Foster City, CA, USA), respectively. Peptide samples were applied to the poly(vinilidenefluoride) membrane in a solution of 30–50% acetonitrile with 0.1% TFA.

2.6. Covalent attachment of M1 protein to thiopropyl Sepharose-6B

In order to change the pH of the M1 solution to neutral or alkaline pH and to prevent the autoaggregation of the protein by the shift the following procedure was used. The M1 protein solution was diluted with 1 *M* Tris–HCl buffer (pH 7.8) to the desired pH value, the obtained solution (500 μl) was concentrated in a SpeedVac concentrator to 100 μl and 150 μl 0.5 *M* Tris–HCl (pH 7.6) in 6 *M* Gu–HCl was added. Disulfide bonds were reduced with β-ME (0.1 *M* β-ME per mole of the thiopropyl Sepharose-6B SH-groups) for 1 h at 37°C. All reactions were carried out in small vessels and all washings and elutions were carried out in pipette tips with a glass wool filter.

Usually, 70 mg dry support (200 μl of swelling suspension gel) was used to attach 0.25 mg (10 nm) M1 protein. Gel was washed with a 30-fold volume of water, a ten-fold volume of 0.1 *M* ammonium bicarbonate buffer (pH 7.8) and finally with a twofold 0.5 *M* Tris–HCl (pH 7.6) in 6 *M* Gu–HCl (coupling buffer). The gel was transferred with a cut pipette into the vessel. Reduced M1 protein (10 nm) was added to a ten-fold molar excess of the support active groups. The gel suspension was gently rotated end over end at room temperature for 4 h. After coupling the gel suspension was washed with two volumes of coupling buffer (the eluate consists of nonbound protein) and additionally with a 20-fold volume of ammonium bicarbonate buffer (pH 7.8) for removing traces of Gu–HCl. This method immobilised 60–80% of the protein.

2.7. Digestion of immobilized M1 protein and detachment of peptides

Trypsin dissolved in 1 *M* ammonium bicarbonate pH 7.8 (1 $mg \cdot ml^{-1}$) was added to the gel suspension to give a final enzyme:substrate ratio of 1:50 (w/w). Hydrolysis was carried out at 37°C for 4 h. Then gel suspension was washed with a two-fold volume of 0.5 *M* Tris–HCl (pH 7.6) in 6 *M* Gu–HCl. The eluate (fraction I, noncysteine-containing peptides) was separated immediately by HPLC.

To detach cysteine-containing peptides, β-ME was added to the suspended gel in a 100-fold molar excess over the support SH-groups and the vessel left for 1 h at room temperature. The suspended gel was washed with a two-fold volume of 0.5 *M* Tris–HCl (pH 7.6) in 6 *M* Gu–HCL. The eluate (fraction II, cysteine-containing peptides) was injected immediately onto the HPLC column.

3. Results and discussion

To study the functional properties of the distinct fragments of M1 protein and to obtain a more refined map of its antigenic determinants, one must perform further enzyme and chemical cleavage experiments and also test synthetic oligopeptides corresponding to the M1 amino acid sequence.

The present linear map of epitopes on the M1 protein of A/WSN/33 influenza virus was determined by binding by four epitope-specific monoclonal antibodies to peptide fragments produced by chemical cleavage with formic acid, N-chlorosuccinimide (NCS) and CNBr [12,13]. These fragments were separated by electrophoresis on a 10–20% gradient polyacrylamide gel containing 7 *M* urea but they are large enough and their number is restricted. Exposure of the M1 protein to formic acid resulted in a 9 kilodalton (kDa) fragment probably extending from amino acid residue 1 to residue 89

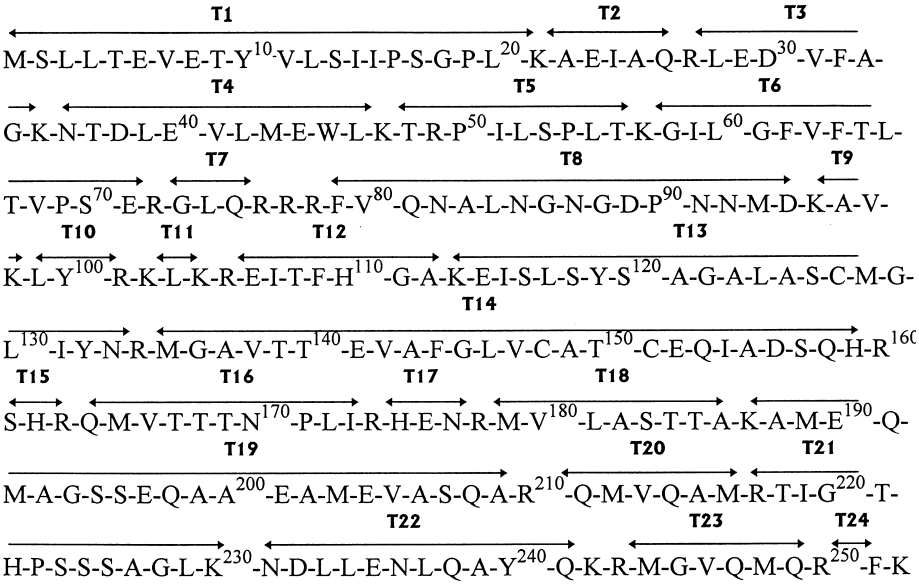

Fig. 1. Amino acid sequence of the M1 protein from the A/Aichi/2/68 (H3N2) strain of influenza virus according to Winter and Fields [14]. The tryptic peptides of the M1 protein are marked as T1–T24.

(Fig. 1) and a 15 kDa fragment at the carboxy-terminal two-thirds [14]. Exposure of the M1 protein to NCS, which cleaves at tryptophan and secondarily at tyrosine or histidine residues resulted in partial cleavage and production of two major peptides and molecular masses of about 10 and 8 kDa [12]. The 8 kDa NCS peptide originated from N-terminal Leu-46 and probably extended to Tyr-119 at its C-terminus. Finally, exposure of the M1 protein to a 50%-fold excess of CNBr in 75% formic acid resulted in production of two major peptides, one of which was identified. This is probably peptide Gly-129–Gln-164. From other peptides generated by protease cleavage of the M1 protein one observes partial cleavage by glytamyl-specific V8 protease resulting in the production of a 16 kDa peptide (residues 1 through 141) [12].

It is not possible to obtain shorter peptides by the direct tryptic hydrolysis because the M1 protein is resistant to enzyme cleavage. A typical chromatogram showing the separation of the reaction mixture is shown in Fig. 2. This chromatogram is different from the usual pattern of trypsin peptides of other proteins. It is seen that the M1 protein is subjected only to limited proteolysis with trypsin (peaks 1–4, Fig. 2). Some enzymes with another proteolytic specificity (V8 glutamine-specific protease, Lys-specific protease) and CNBr were used to cause M1 protein cleavage, but the results were not as satisfactory as for the trypsin treatment. In these experiments, the M1 protein was denatured, disulfide-bonds were reduced and sequentially alkylated before hydrolysis.

To increase the M1 protein susceptibility to trypsin in order to obtain more fragments we used the specific reversible covalent attachment of M1 to a solid-phase to stretch it. This method was developed for the specific isolation and also identification of S–S-peptides and their position in large and complex proteins [7].

M1 protein has three cysteinyl residues [15]. Sulfhydryl groups are highly susceptible to oxidation to the disulfide by atmospheric oxygen in the presence of iron salts or by other mild oxidizing agents. Thus any disulfide crosslinks in the M1 protein were cleaved by a reducing agent. In several proteins

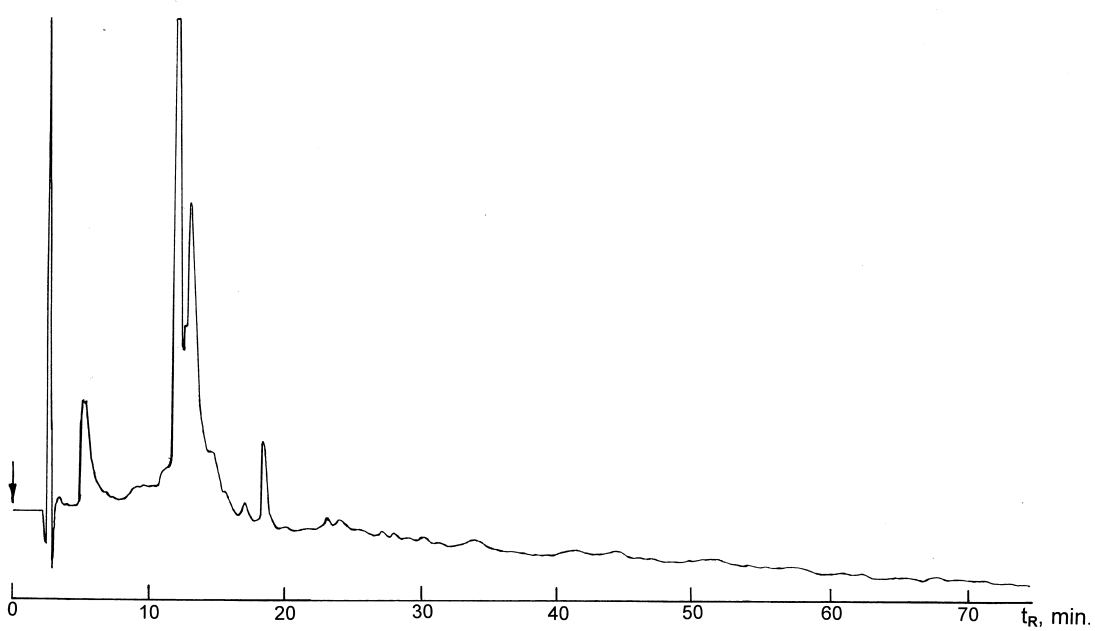

Fig. 2. RP-HPLC separation of the M1 protein tryptic peptides obtained under ''standard'' proteolysis. Elution conditions: first 3 min 0.1% TFA in water, then a linear gradient up to 50% of acetonitrile in 60 min and then up to 60% of acetonitrile in 10 min. Spectrophotometric detection at 215 nm.

SH-groups are not accessible to solvent, and thus a denaturing agent was added to the coupling mixture. Both urea and Gu–HCl could be used. However, since Gu–HCl is in general more efficient it was used routinely.

Immobilization and enzymic digestion of the covalently bound M1 protein were performed essentially as described previously [10]. Typical chromatograms of the separations of the noncysteine-containing and cysteine-containing peptides are shown in Figs. 3 and 4. The peptides were identified by amino acid analysis and partial sequencing. The comparison of the amino acid composition of the isolated peptides with a due account of the sequences of the first three (in the case of cysteine-containing peptides we determined sequences of ten more residues) permits us to suggest their location in the M1 polypeptide chain (Fig. 1). These results show that specific reversible covalent attachment to a solid-phase as a means of isolating peptides to date is apparently the most effective method for enzymatic fragmentation and analysis of highly hydrophobic proteins, such as membrane proteins. In addition, this method provides a new way to investigate the functional properties of distinct domains of viral thiol proteins by allowing their separation.

4. Conclusion

A rapid and specific method for the isolation of thiol-containing peptides from large proteins by thiol–disulphide exchange on a solid support has been applied to the hydrophobic membrane M1 protein as a tool to facilitate its fragmentation. When the M1 protein was immobilized on the insoluble activated support thiopropyl Sepharose-6B it became susceptible to trypsin hydrolysis. Twenty-four tryptic peptides were isolated and identified. Further study of their immunochemical properties may be performed.

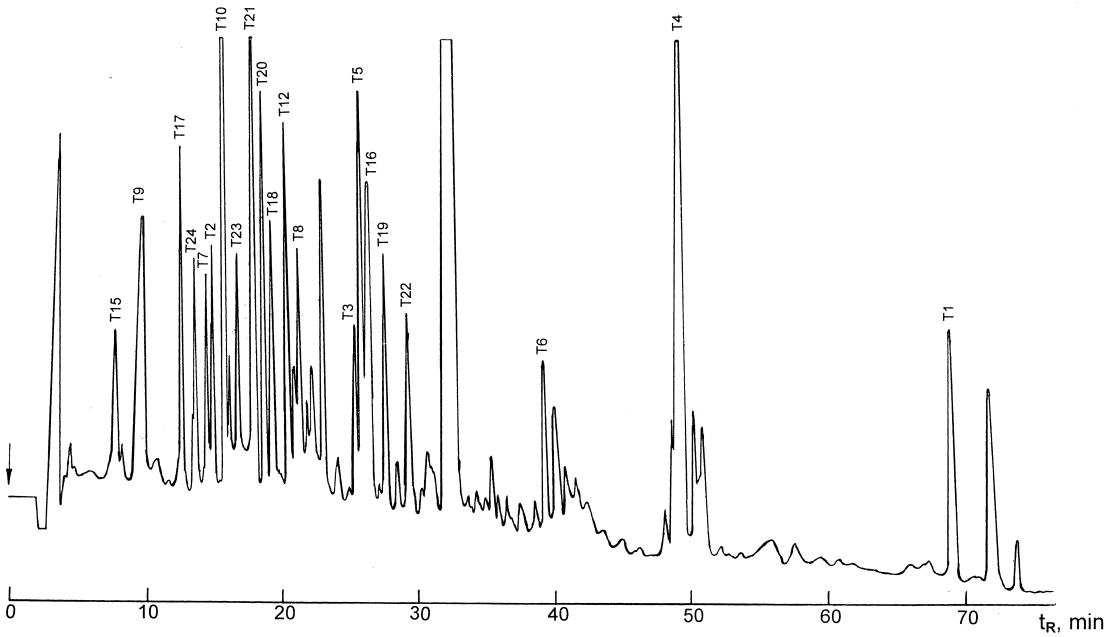

Fig. 3. Separation of tryptic noncysteine-containing peptides of M1 protein immobilized on support. Elution conditions: first 3 min 0.1% TFA in water, then a linear gradient up to 50% of acetonitrile in 60 min and then up to 60% of acetonitrile in 10 min. Spectrophotometric detection at 215 nm.

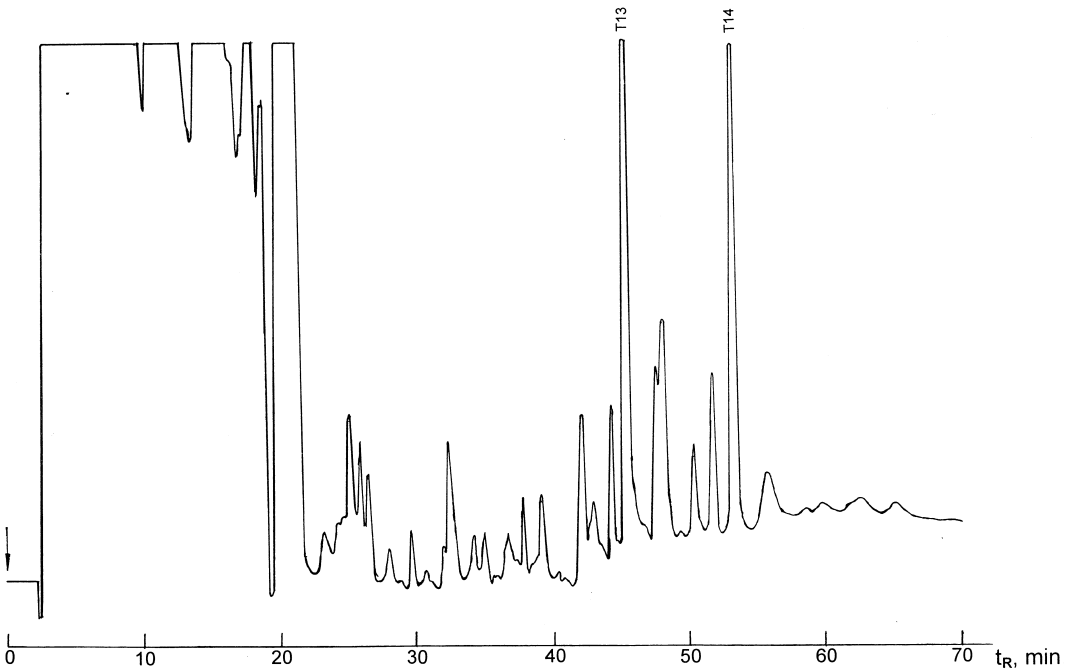

Fig. 4. Separation of tryptic cysteine-containing peptides of M1 protein immobilized on support. Elution conditions are as in Fig. 3. Spectrophotometric detection at 215 nm.

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References

[1] I.T. Schulze, Adv. Virus Res. 18 (1973) 1–56.
[2] W.G. Laver, Adv. Virus Res. 18 (1973) 57–104.
[3] R.A. Lamb, in: R.M. Krug (Ed.), The Influenza Viruses, Plenum Press, New York, 1989, pp. 1–87.
[4] O.P. Zhirnov, Virology 176 (1990) 274–279.
[5] O.P. Zhirnov, Virology 186 (1992) 324–330.
[6] O.P. Zhirnov, M. Ohuchi, V.S. Akvayans, A.V. Ovcharenko, C.D. Klenk, Mol. Biol. (Moscow) 31 (1997) 137–143.
[7] T.A. Egorov, A. Svenson, L. Ryden, J. Carlson, Proc. Natl. Acad. Sci. 72 (1975) 3029–3033.
[8] O.P. Zhirnov, A.V. Ovcharenko, A.G. Bucrinskaya, J. Gen. Virol. 66 (1985) 1633–1638.
[9] U.K. Laemmli, Nature (London) 227 (1970) 680–685.
[10] Ts.A. Egorov, J. Protein Chem. 9 (1990) 281.
[11] A. Tsugita, J.-J. Scheffler, Eur. J. Biochem. 124 (1982) 585–588.
[12] Z. Ye, N.W. Baylor, R.R. Wagner, J. Virol. 63 (1989) 3586–3594.
[13] D. Bucher, S. Popple, M. Baer, A. Mikhail, Y.-F. Gong, C. Whitaker, E. Paoletti, A. Judd, J. Virol. 63 (1989) 3622–3633.
[14] G. Winter, S. Fields, Nucleic Acids Res. 8 (1980) 1965–1974.
[15] Z. Ye, R. Pal, J.W. Fox, R.R. Wagner, J. Virol. 61 (1987) 239–246.