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# Identification of the cap binding protein of influenza virus

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ABSTRACT

The presence of a cap binding protein in influenza virus PR8 has recently been demonstrated by photoaffinity labelling with the cap-analogue ($\gamma[^{32}P]$-[4-(benzoylphenyl)methylamido]-7-methylguanosine 5'-triphosphate). This paper describes the identification of the labelled protein using two-dimensional gel electrophoresis. The protein is shown to be PB2, the smaller of the two basic P proteins in the polymerase complex.

## INTRODUCTION

mRNA synthesis of influenza virus is initiated by primers generated from host cell capped RNA. The mechanism of the priming event has been extensively investigated (1,2). A viral endonuclease cleaves a capped fragment of 10 to 13 nucleotides from the 5' end of host RNA, which is subsequently elongated to form a copy of the negative strand genomic RNA. Transcription of viral mRNA is terminated prematurely and poly (A) is added. The formation of plus-strand RNA during viral replication follows a different route, giving rise to full-length copies of the viral RNA. The molecular mechanism regulating the different modes of transcription is still unknown.

Recently two approaches have been described to unravel the role of individual proteins in the viral transcriptase complex. Ulmanen et al. (3) have employed short-wave UV crosslinking between radioactive mRNA and influenza virus cores of strain WSN. This method utilizes the photoreactivity of natural nucleotides which can be activated by irradiation at 254 nm. UV-crosslinking has been employed previously to study protein-nucleic acid neighbourhoods on ribosomes, but is known to lead to rapid inactivation of ribonucleoprotein complexes (4). The



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photoaffinity technique described by our group uses a small molecular weight cap analogue, $\gamma[^{32}P]$-[4-(benzoylphenyl)methyl-amido]-7-methylguanosine-5'-triphosphate(BP-$m^7$GTP) containing a photoreactive benzophenone-moiety (5). The activation of the aromatic ketone residue occurs at 320 nm. Light of this wavelength is not absorbed by proteins or by nucleotides occuring in mRNA. The photoreaction can therefore be performed under conditions where the functional integrity of the complex is preserved. The use of a small molecular weight compound furthermore guarantees that covalent bond formation takes place close to the site of specific recognition, i.e. the cap structure. Using the two-dimensional gel system the labelled protein is identified as PB2, the smaller of the two basic P proteins.

## MATERIAL AND METHODS

### Synthesis of $\gamma[^{32}P]$-BP-$m^7$GTP

$\gamma[^{32}P]$GTP was prepared by a modification of the method of Glynn and Chappel (6), starting from 20 mCi of carrier free $[^{32}P]$ orthophosphate (Radiochemical Centre, Amersham) and 300 nmoles of GTP. GTP was separated from inorganic phosphate on a DEAE cellulose column equilibrated with 50 mM triethylammoniumbicarbonate pH 7.8 (TEAB). After washing with 50 mM TEAB, inorganic phosphate was eluted with 150 mM TEAB, GTP with 500 mM TEAB. Peak fractions were identified by thin layer chromatography, pooled and lyophilized (System A: GTP, Rf = 0.2; $PO_4^{3-}$, Rf = 0.73). $\gamma[^{32}P]$GTP was methylated with dimethylsulfate (7, 8) and separated from residual inorganic phosphate and GTP on a DEAE cellulose column equilibrated with 50 mM TEAB. $m^7$GTP was eluted with 200 mM TEAB; GTP with 500 mM TEAB. Peak fractions were pooled and lyophilized (thin layer chromatography:System B: GTP, Rf = 0.09; $m^7$GTP, Rf = 0.51; $PO_4^{3-}$, Rf = 0.65). $\gamma[^{32}P]m^7$GTP was dissolved in 12 µl 0.1 M pyridiniumchloride (pH 5.7); 6.4 mg N-cyclohexyl-N'-[β-(N-methylmorpholino-ethyl]-carbodiimide-p-toluenesulfonate in 48 µl water was added, and the mixture incubated at room temperature for 10 min (9). 7.6 mg 4-aminomethyl-benzophenone (BP, kindly provided by Dr. A. Haslinger) in 60 µl dimethylformamide was added, and the incubation continued for an additional 3 hrs. The reaction mixture was di-

luted tenfold and loaded on to a DEAE cellulose column equilibrated with 10 mM TEAB. The column was extensively washed with 10 mM TEAB and a gradient of 50 to 500 mM TEAB was applied. Fractions containing BP-$m^7$GTP were identified by thin layer chromatography (System B: BP-$m^7$GTP, Rf = 0.56; $m^7$GTP, Rf = 0.51. System C: BP-$m^7$GTP, Rf = 0.43; BP, Rf = 0.70). Pooled fractions were adsorbed on a SEP-PAK C 18 cartridge (Waters Ass. Inc., Milford, USA). After washing with 200 mM TEAB, BP-$m^7$GTP was eluted with 50 % ethanol. Specific activities of different preparations of [$^{32}$P] BP-$m^7$GTP varied between 30 and 50 Ci $mmol^{-1}$. The molar extinction coefficient of BP-$m^7$GTP is 28.5 x $10^3$ l. $mol^{-1}$. $cm^{-1}$. It is easily cleaved by acid hydrolysis with 0.1 M HCl for 1 hr at 37° C into $m^7$GTP and BP.

Column and thin layer chromatography

All ion exchange column chromatographies were carried out on columns 0.5 x 10 cm filled with DE-52 cellulose (Whatman). Thin layer chromatography: System A: PEI cellulose Cel 400 plates (Macherey & Nagel, Düren, Germany), 0.75 M potassium phosphate, pH 3.5. System B: same as A, but 0.5 M potassium phosphate. System C: Silica gel plates F 1500 LS 254 (Schleicher & Schüll, Dassel, Germany) chloroform: methanol:water (20/30/7 v/v).

Polyacrylamide gel electrophoresis

Non equilibrium pH gradient electrophoresis was performed at 2500 V hrs using gel tubes with an inner diameter of 1 mm (10). The second dimension was carried out on 10 % gels (150 x 170 x 0.8 mm) in SDS buffer (5). The gels were silver-stained following the method of Merril (11).

Transcriptase assay and photoaffinity labelling

The conditions of the transcriptase assay and of the photoaffinity labelling were as described (5). The incorporation of [$^3$H]UTP/mg of influenza virus protein was in the range of 550 - 1270 pmol per hr for the globin mRNA and the ApG primed reaction. The time of irradiation in the photoaffinity labelling experiments was 5 min. Autoradiographs of gels of labelled proteins were scanned on a Beckman DU 8 spectrophotometer.

RESULTS

In order to investigate the influence of the irradiation on the transcriptase activity, detergent treated virions were

irradiated with a high pressure mercury lamp at wavelengths >300 nm for various time periods (Fig. 1). The experimental conditions were identical to those used for the photoaffinity reaction (5). After the times indicated aliquots were removed and assayed for enzymatic activity using globin mRNA as primer (5). It can be seen that no significant inactivation occured during the time of irradiation (5 min) employed in the photoaffinity labelling. Even prolonged irradiation had only a marginal effect on the incorporation of [$^3$H]UTP into RNA.

In the one-dimensional SDS-polyacrylamide slab gel electrophoresis system which is usually employed, all influenza virus proteins are well separated except for P2 and P3, which are very similar in size. Depending on the particular gel system used, P2 and P3 proteins can also be found to comigrate or can even be reversed in order (10). Fortunately the two proteins differ in their isoelectric points, thus allowing a separation on an isoelectric focusing gel. The two-dimensional gel system described by Horisberger (10) combines nonequilibrium pH gradient electrophoresis in the first dimension with a separation according to size, in the presence of SDS, in the second dimension.

Fig. 2 shows the results of the two-dimensional gel electrophoresis of the proteins from the transcriptase complex photolabelled with [$^{32}$P]BP-m$^7$GTP. Fig. 2a presents the protein pattern as obtained by silver-staining (11). Besides the major

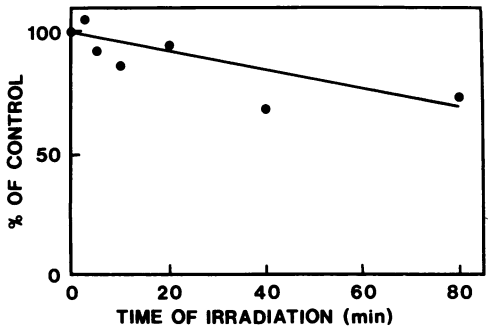


Fig. 1. Effect of irradiation on viral transcriptase activity. Virus was treated with NP-40 and irradiated at 98 mW/cm$^2$ with a 500 W super high-pressure mercury lamp using a WG 320 filter at 4° C. Aliquots corresponding to 30 µg of viral protein were removed at the times indicated. The polymerase activity was determined by the incorporation of [$^3$H]UTP into trichloroacetic acid precipitable material using globin mRNA as a primer, as described (5).

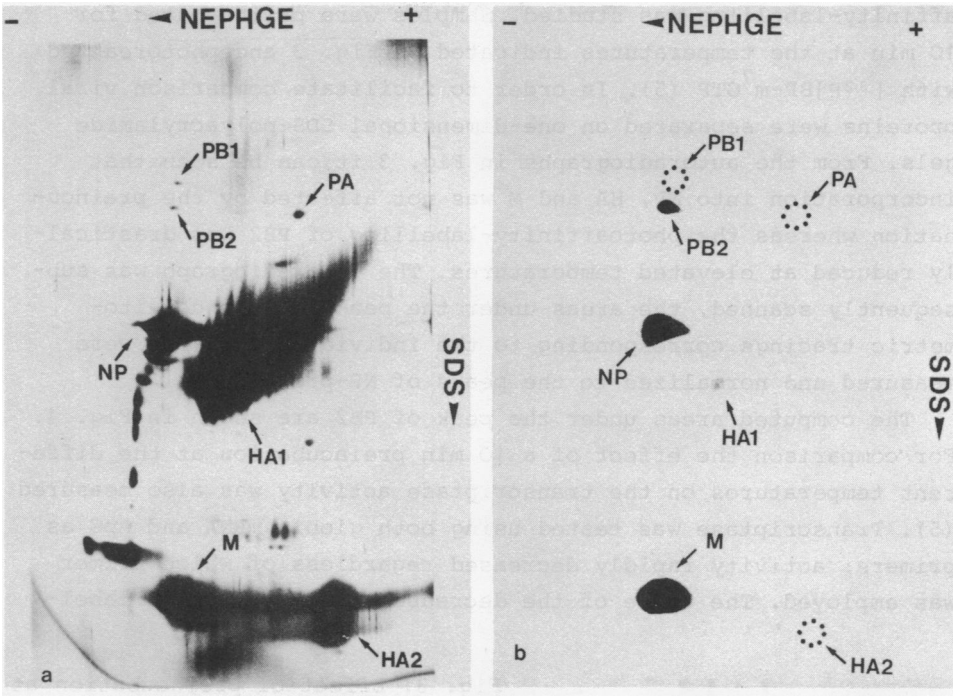


Fig. 2. Two-dimensional separation of [$^{32}$P]BP-m$^{7}$GTP labelled viral proteins. 40 µg of photoaffinity labelled viral protein was applied. A: Silver-stained gel; B: corresponding autoradiograph.

viral proteins the spots of the P proteins are clearly seen in the positions described by Horisberger (10). Rather than using the conventional nomenclature we have followed the suggestion of Ulmanen et al. (3) and have labelled the large basic P protein, PB1, the small basic P protein, PB2, and the acidic P protein, PA. Fig. 2b shows the corresponding autoradiograph. Out of the three P proteins, only the small basic P protein (PB2) is labelled. In the second dimension of the gel system employed, PB2 migrates somewhat more slowly than PA. This is in agreement with the previous identification of the labelled P protein as P2 on the one-dimensional SDS slab gel system (5). Again [$^{32}$P]BP-m$^{7}$GTP was also found to be incorporated into other proteins, such as NP, M etc. However, this labelling is nonspecific as shown previously by the lack of competition with cap analogues (5).

Next, the influence of thermal inactivation on the photo-

affinity-labelling was studied. Samples were preincubated for 10 min at the temperatures indicated in Fig. 3 and photoreacted with [$^{32}$P]BP-m$^{7}$GTP (5). In order to facilitate comparison viral proteins were separated on one-dimensional SDS-polyacrylamide gels. From the autoradiographs in Fig. 3 it can be seen that incorporation into NP, HA and M was not affected by the preincubation whereas the photoaffinity-labelling of PB2 was drastically reduced at elevated temperatures. The autoradiograph was subsequently scanned, the areas under the peaks of the densitometric tracings corresponding to the individual proteins were measured and normalized to the peaks of NP-protein.

The computed areas under the peak of PB2 are shown in Fig. 4. For comparison the effect of a 10 min preincubation at the different temperatures on the transcriptase activity was also measured (5). Transcriptase was tested using both globin mRNA and ApG as primers; activity rapidly decreased regardless of which primer was employed. The curve of the decrease in photoaffinity-label-

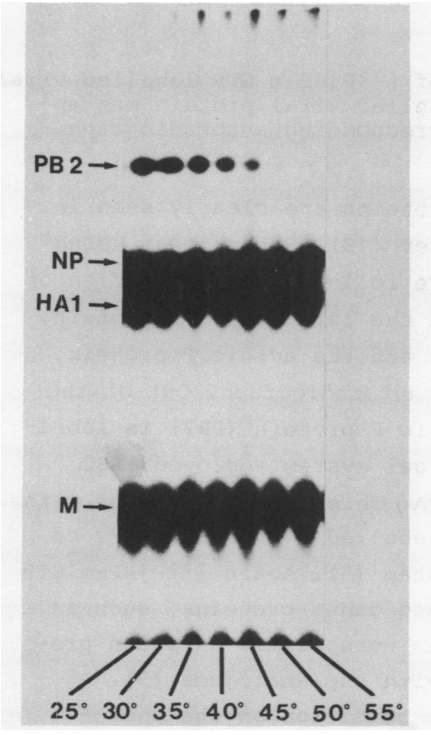


Fig. 3. Effect of preincubation at different temperatures on photoaffinity-labelling. NP-40 treated virus was preincubated for 10 min at the temperatures indicated. Aliquots (corresponding to 15 µg of viral protein) were subjected to photoaffinity-labelling with [$^{32}$P]BP-m$^{7}$GTP. Proteins were separated by one-dimensional gel electrophoresis in SDS-buffer (5) and autoradiographed.

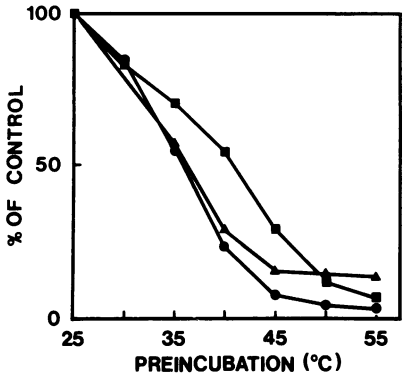


Fig. 4. Comparison of the effect of preincubation at various temperatures on transcriptase activity and on specific photoaffinity-labelling of PB2. NP-40 treated virus was incubated for 10 min at the temperatures indicated. Aliquots (corresponding to 30 µg of viral protein) were removed and transcriptase assays were performed using either ApG (●-●) or globin mRNA (▲-▲) as primers. Autoradiograph of the gel shown in Fig. 3 was scanned and densitometric tracings were normalized to the peak of NP (5). Areas under the peak of PB2 corresponding to [$^{32}$P]BP-m$^7$GTP incorporated were measured (■-■).

ling of PB2 follows the overall shape of the thermal inactivation of the enzymatic activity. When comparing the curves it should be noted that the enzymatic assay and the affinity-labelling measure two different aspects of the transcriptase activity. Whereas the incorporation of radioactive UTP depends both on initiation and elongation of the RNA chains, affinity-labelling depends solely on the binding of the cap structure. Nevertheless, photoaffinity-labelling of PB2 is completely prevented by heat inactivation of the transcriptase complex. The effect of elevated temperatures complements the earlier study on the denaturation of transcriptase by a strong detergent : pretreatment of the complexes with SDS specifically abolished labelling of PB2 without reducing incorporation of the label into the other proteins (5).

## DISCUSSION

The importance of the structural and functional integrity of the viral transcriptase for specific photoaffinity-labelling is illustrated by the inhibition of incorporation of [$^{32}$P]BP-m$^7$GTP into PB2 upon preincubation at elevated temperatures (Fig. 3) and upon exposure to SDS, which disintegrates the complex (5). It is therefore important to ensure that the enzymatic activity is not destroyed by the irradiation itself. Short wave UV-light as used by Ulmanen et al.(3) is known to lead to several side reactions, such as photooxidation and other photoreactions, which damage

nucleic acids as well as proteins. Under the conditions of the photoreaction with BP-$m^7$GTP as employed in this study, damage to the transcriptase complex is negligible (Fig. 1), assuring that the photoaffinity labelling takes place on active complexes.

The thermal inactivation curves of the transcriptase activities primed by globin mRNA and by the artificial primer ApG respectively follow each other rather closely (Fig. 4). It is therefore unlikely that the heat inactivation primarily destroys the cap-binding activity of the transcriptase but rather seems to affect some other function of the complex. This also explains the discrepancy in the curves between the decrease in photoaffinity-labelling and the decline of enzymatic activity (Fig. 4).

The apparent difference in the labelling of HA1 between the two-dimensional system in Fig. 2 and the one-dimensional system shown in Fig. 3 also deserves a comment: even though differences in the nonspecific labelling of the various viral proteins have been observed in different preparations, the low density in HA1 in Fig. 2b seems to be mainly due to the spread of HA1 over a relatively large area as compared to the other viral proteins (Fig. 2a). This is frequently observed with glycosylated proteins.

In our previous paper the cap-binding protein of influenza virus PR8 was identified as P2 by one-dimensional slab gel electrophoresis in SDS buffer (5). In the second dimension of the two-dimensional system (Fig. 2), PB2 indeed migrates slower than PA, thus confirming our previous assignment. Furthermore, this agrees with the designations of P proteins on two-dimensional gels recently described by Horisberger and de Staritzky (12). Short wave UV irradiation at a high light dosage of core complexes of influenza virus strain WSN by Ulmanen *et al.* (3) photocrosslinked cap-labelled alfalfa mosaic virus RNA with P3. This discrepancy, however, seems to be due to the inversed relative mobilities of PB2 and PA in the second dimension of the two-dimensional system and not due to the labelling of two functionally distinct viral proteins. In their two-dimensional gel system the labelled P3 migrates in the position of PB2. Changes in the migrational pattern of the P-proteins on SDS-gels have been noted previously by Horisberger (10). Using apparently identical gel conditions, alterations in the migrational

distances of PB2 and PA in the second dimension of the gel have been observed even with the same strain of influenza virus (NWS), which resulted in an inversion of the bands. The reasons for this phenomenon are unclear. In addition the cap-binding proteins of two more strains of influenza A virus, A/FPV/Rostock/34 and A/FPV/Dutch/27(Dobson), have recently been identified as P2 and P3, respectively, by one-dimensional gel electrophoresis (13). When comparing the two strains on two-dimensional gels, however, the cap-binding proteins migrated as the small basic P, i.e. PB2 (Penn, C.R., Blaas, D., Kuechler, E. and Mahy, B.W.J. unpublished).

In vivo studies of ts-mutants of influenza A with a defect in the synthesis of complementary RNA have shown that the lesions are located in the genes coding for the two basic P-proteins (14; P. Palese, personal communication). A comparison of in vitro transcription using transcriptase complexes prepared from a set of temperature-sensitive mutants of influenza A FPV/Rostock/34 has demonstrated that at the restrictive temperature two mutants in P2 (ts9, ts44) were defective in globin mRNA primed RNA synthesis, but were permissive with ApG as a primer (15,16). This indicates that P2 of strain Rostock is important for priming transcription with natural messenger RNA. Thus biochemical as well as genetic data support the conclusions drawn from the affinity-labelling experiments.

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