An acidic region of the 89K murine cytomegalovirus immediate early protein interacts with DNA

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The product of the iel gene, the regulatory immediate early protein pp89 of murine cytomegalovirus (MCMV), interacts with core histones, which can mediate the association of pp89 with DNA. We report the capacity of pp89 to interact directly with DNA in the absence of cellular proteins. After separation of proteins by SDS–PAGE, pp89 bound ds- and ssDNA, with a preference for ssDNA. Binding to specific DNA sequences in the MCMV genome was not detected. The DNA-binding region of pp89 was located to amino acids 438 to 534 by analysis of deletion mutants expressed as β-galactosidase or TrpE fusion proteins. This region is identical to the highly acidic C-terminal region spanning amino acids 424 to 532. The human cytomegalovirus IE1 protein, which contains a similar extended C-terminal acidic region, does not react with DNA under the same experimental conditions.

Introduction

Gene expression during the course of the murine cytomegalovirus (MCMV) replication cycle is temporally controlled and regulated in a cascade fashion (Keil et al., 1984). At least three classes of MCMV genes, immediate early (IE or α), early (E or β), and late or γ can be differentiated. Three major IE genes, iel, ie2 and ie3, have been identified (Keil et al., 1987a, b; Messerle et al., 1991). Genes iel and ie3 share the same promoter and are separated from gene ie2 by a regulator region with enhancer elements. Although MCMV gene ie2 has no counterpart in human cytomegalovirus (HCMV) IE genes (Messerle et al., 1991), the structural organization of the HCMV IE1/IE2 gene complex is comparable to that of MCMV iel/ie3 (Keil et al., 1987a; Stinski et al., 1983). MCMV iel and HCMV IE1 products are encoded by exons 2, 3 and 4, and MCMV ie3 and HCMV IE2 products by exons 2, 3 and 5. Sequence comparison of these proteins revealed similarity only between MCMV IE3 and HCMV IE2 amino acid sequences encoded by the respective exons 5 (Messerle et al., 1992). Similarity between MCMV and HCMV IE1 proteins is restricted to the highly acidic C-terminal regions encoded by exons 4 (Keil et al., 1987a).

IE proteins are required for the trans-activation of E gene promoters. Activation of heterologous viral and cellular promoters by the MCMV IE1 protein pp89 has been demonstrated by transfection of the iel gene (Koszinowski et al., 1986; Schickedanz et al., 1988). This is in contrast to the inability of the IE1 protein expressed in transfected cells to activate the MCMV E gene promoter e1, which requires the presence of ie3 gene products (Bühler et al., 1990). However, transient expression experiments have revealed that E gene activation by the IE3 protein can be augmented by iel gene products (Messerle et al., 1992). No function in transcriptional regulation has been assigned to the ie2 product (Koszinowski et al., 1986).

MCMV IE1 proteins interact with chromatin and this property could play a role in gene activation. pp89 and its proteolytic cleavage product pp76 bind avidly to isolated core histones and a histone-binding region has been located to amino acids 71 to 415, but not to the region homologous to histone H2B (Münch et al., 1991). When proteins in high salt extracts of infected cells are analysed by chromatography on DNA-cellulose, histones are required for the interaction of pp89 with DNA (Münch et al., 1988). No association of pp89 with cellular DNA in dividing cells could be detected. This was explained by the higher affinity of pp89 for isolated histones than for DNA-bound histones. This finding is in contrast to that for the HCMV IE1 protein, which has been reported to interact with metaphase chromosomes; sequences encoded by exon 4 contribute to this activity (LaFemina et al., 1989).

We now demonstrate that pp89 has an intrinsic capacity to interact directly with DNA. We have defined a domain of pp89 essential for DNA binding using a set of pp89 fusion proteins blotted onto nitrocellulose and incubated with DNA probes, which were detected by autoradiography. We report that pp89 binds DNA,
preferentially ssDNA, in a sequence-independent manner. The DNA-binding region was located within the highly acidic C-terminal region of exon 4-encoded sequences.

**Methods**

Virus and cells. MCMV (mouse salivary gland virus, strain Smith, ATCC VR-194) was used for infection of BALB/c mouse fibroblasts (MEFs) as described by Keil et al. (1984).

Western blot analysis. MCMV IE polypeptides were visualized by probing filters with monoclonal antibody (MAb) 6/20/1 and with antisera raised against peptides of pp89 as described (Keil et al., 1985; Münch et al., 1991). For detection of HCMV IE1 proteins a mixture of human HCMV-positive sera was used.

Recombinant plasmids and expression of pp89 and pp89 peptides in Escherichia coli. β-Galactosidase (β-gal)-pp89 fusion proteins were generated by ligation of MCMV iel gene sequences encoding amino acids 1 to 99, and deletion mutants affecting amino acids 20 to 134, 50 to 152, 137 to 248, 275 to 475, 251 to 595, 154 to 595 and 100 to 595, respectively (Volkmer et al., 1987), to the Smal site of the expression vector pROS (Ellinger et al., 1989) (obtained from G. Jahn, Institute for Virology, Erlangen, Germany). E. coli BMH 71/18 was transformed with the recombinant constructs, grown to an optical density of 0.6 at 550 nm and induced for 5 h with 1 mM IPTG. For expression of β-gal–HCMV IE1 fusion proteins the IE1 open reading frame was ligated to the HindIII site of the pROS vector, and transformation and induction were done as described above. TrpE fusion proteins containing amino acids 1 to 99, 122 to 153, 250 to 309, 310 to 438, 438 to 534, 468 to 492 and 532 to 595 of pp89 were generated by fusion of the corresponding MCMV iel gene sequences to the Smal site of the pATH 2 shuttle vector (Dieckmann & Tzagloff, 1985; Spindler et al., 1984). E. coli C600 was transformed with these plasmids, grown to an optical density of 0.2 at 550 nm and induced for 5 h with 3-β-indoleacrylic acid (10 μg/ml).

Labelling of MCMV DNA. MCMV DNA was either end-labelled with [γ-32P]ATP and T4 polynucleotide kinase, or labelled with [α-32P]dCTP and the Klenow fragment of DNA polymerase by random oligonucleotide-primed synthesis (Feinberg & Vogelstein, 1983).

Protein blotting and identification of DNA-binding proteins. Proteins from infected cells were separated by SDS-PAGE and transferred to nitrocellulose as described previously (Münch et al., 1988). Proteins expressed by bacteria were treated in the same way after sonication for 1 min at 100 J/s (Brown Sonifier B-12). After transfer, the nitrocellulose was incubated in 1% bovine serum albumin in PBS for 2 h at 4°C, cut in strips and briefly rinsed in PBS. If not otherwise indicated strips were incubated in binding buffer (100 mM-NaCl, 20 mM-Tris–HCl pH 7.5, 10% glycerol) and 32P-labelled MCMV DNA for 14 h at 4°C. Unbound DNA was removed by washing the strips three times for 5 min in binding buffer at 23°C prior to exposure to X-ray film.

**Results**

pp89 interacts directly with DNA

Previous studies using chromatography of MCMV-infected cell extracts on DNA-cellulose have demonstrated the interaction of the major MCMV IE proteins with DNA. Both pp89 and pp76, a processed product of pp89 (Keil et al., 1985), bind core histones with high affinity and core histones are involved in the interaction of pp89 with DNA (Münch et al., 1988). However, it remained unclear whether pp89 contains an intrinsic DNA-binding region that is perhaps activated subsequent to the interaction with histones or whether pp89 is kept in DNA–histone complexes only by its affinity for histones.

To study the intrinsic DNA-binding activity of IE proteins, the contribution of cellular proteins to this reaction had to be excluded. We used the method of DNA binding of proteins separated by SDS–PAGE after transfer to nitrocellulose (Southwestern assay), which has been successfully applied to determine the interaction of wild-type proteins, protein fragments and fusion proteins with DNA, with or without preference for specific DNA sequences (Lenz et al., 1990; Moreland et al., 1991; Tsujimoto et al., 1991; Inoue et al., 1991). MCMV-infected cell extracts were separated by SDS–PAGE and proteins were transferred to nitrocellulose. After renaturation the immobilized proteins were incubated with DNA 32P-labelled according to the method of Feinberg & Vogelstein (1983) (Fig. 1, lanes 2 to 6).
position of the IE proteins pp89 and pp76 was determined by Western blotting (lane 1).

pp89 and pp76 were both found to bind DNA and clearly represented the most prominent DNA-binding proteins in extracts of infected cells, although they are not the most abundant cellular proteins, as demonstrated by Coomassie blue staining (lane 7). The other prominent DNA-binding proteins migrating at 32K, 17K and 15K represent the cellular histones. DNA-binding of the IE proteins decreased with increasing ionic strength, and the affinity of pp89 for DNA was abolished at 0.4 to 0.6 M-NaCl (lanes 5 and 6). The DNA-binding capacity of pp76 was found to be reduced but not completely abolished at 0.6 M-NaCl. The properties of pp76 were not followed further because this protein represents a degradation product of pp89, and its relative abundance is variable and mainly dependent upon cell lysis and extraction procedures. These results show that isolated pp89, after separation from cellular proteins, binds directly to DNA, and that the binding properties are comparable to those of histones, the abundant cellular DNA-binding proteins.

**pp89 preferentially binds ssDNA**

We wished to determine which regions of pp89 contribute to DNA binding and prepared as a first step a pp89-β-gal fusion protein containing the complete pp89 sequence. Bacterial extracts were used for DNA binding studies. It was expected that in the experiment shown in Fig. 1 protein would bind to ssDNA because labelling of DNA by random oligonucleotide-primed synthesis results mainly in ssDNA probes. However, due to the renaturation of the partially complementary single strands a selective reaction with dsDNA in the solution was not excluded. To test for selective binding of pp89 to ss- or dsDNA, end-labelled DNA fragments were used for binding studies, either as native dsDNA or as ssDNA after heat denaturation (Fig. 2). Single-stranded DNA was found to be bound effectively at 100 mM- and 200 mM-NaCl, and with a reduced affinity at 300 mM-NaCl (Fig. 2a, lanes 1, 2 and 3). At a concentration of 400 mM-NaCl, binding of ssDNA was weak (lane 4). Binding of dsDNA was weaker than that of ssDNA at 100 mM- and 200 mM-NaCl and further reduced at 200 mM-NaCl (Fig. 2b, lanes 1 and 2). At higher NaCl concentration, no dsDNA was bound (lanes 3 and 4). Two conclusions were drawn from these results. First, pp89 can bind both ss- and dsDNA, but the affinity for ssDNA is greater. Second, the affinity for ssDNA of the authentic pp89 and of the β-gal–pp89 fusion protein is comparable (compare Fig. 1 and Fig. 2a).

To investigate whether pp89 also binds RNA, ssDNA binding studies were performed in the presence of a 20-, 100- or 500-fold excess of RNA (Fig. 2c, lanes 2 to 4). No inhibition of DNA binding could be observed even at the highest RNA concentration, regardless of whether bacterial or eukaryotic RNA was used for competition. As the same concentration of unlabelled DNA competed effectively with DNA binding (see Fig. 3), we concluded that pp89 does not interact with RNA.

**No sequence specificity of pp89 binding**

The DNA-binding activity of pp89 and pp76 was comparable to that of the most prominent cellular DNA-
binding proteins (Fig. 1) and it could be hypothesized that specific sequences of the labelled MCMV DNA were recognized. To test this hypothesis, an excess of either specific or non-specific DNA was used to compete for DNA binding by pp89 (Fig. 3). Binding of labelled MCMV DNA was partially inhibited at a 20-fold excess, effectively reduced by a 100-fold excess and completely abolished at a 500-fold excess of unlabelled MCMV DNA (Fig. 3a, lanes 1 to 4). Competition also occurred when calf thymus DNA was used as the competitor (Fig. 3b, lanes 2 to 4). The same results were obtained using several DNA fragments of the MCMV genome, including the region represented by the iel gene promoter and enhancer sequences, and the el gene region (data not shown). Taken together, competition was always found to be comparable when either MCMV DNA fragments or calf thymus DNA were used for competition. These results indicate that the DNA-binding activity identified by these criteria is not due to a sequence-specific DNA-binding property of pp89.

Localization of the DNA-binding region of pp89

To define the DNA-binding region of pp89 a series of deletion mutants were expressed either as β-gal or TrpE fusion proteins and tested for their DNA affinity (Fig. 4).

To study the expression of the different gene constructs and to identify the individual proteins the extracts of bacterial cells were separated by SDS-PAGE and proteins were stained with Coomassie blue (Fig. 4a) or were reacted with MAb 6/20/1 or antisera raised against peptides of pp89 (Münch et al., 1991) (not shown). Extracts of bacterial cells expressing the β-gal (Fig. 4b, lanes 1 to 8) or the TrpE proteins (Fig. 4b, lanes 9 to 16) contained only one bacterial protein of Mr, 170K with significant DNA-binding properties. The reactivity of this protein was used as an internal standard, unrelated to protein abundance detected by Coomassie blue staining and immunoblotting, to evaluate the DNA affinity of the individual chimeric proteins containing pp89 sequences. No DNA affinity of the β-gal (lane 1) and TrpE proteins (lane 9), which have Mr's of 44K and 41K, respectively, was detected. The β-gal fusion proteins containing pp89 and mutants A, N and F, which are characterized by deletions in the N-terminal region (for sequence details see Fig. 4c), all bound DNA strongly (Fig. 4b, lanes 2 to 5). It is apparent that several fusion proteins were not stable and that some, but not all, degradation products were still capable of binding DNA. Deletion mutant C, which lacks amino acids 274 to 476, showed reduced DNA-binding activity (lane 6) and deletion mutant H, containing only the first 250 amino acids of pp89, lacked this activity altogether (lane 7). These results indicated to us that the first 250 amino acids of pp89 can be deleted without loss of function and have no activity on their own. Since even amino acids 274 to 476 can be deleted without complete loss of binding activity, the DNA-binding region should be located in the C-terminal third of pp89.

In good agreement with this explanation was the finding that the regions expressed by mutants H, J and 5', which contain the N-terminal 250, 154 and 99 amino acids, respectively, and amino acids 122 to 153 expressed by mutant H2B did not bind DNA (Fig. 4b, lanes 7 to 9). Of the deletion mutants containing amino acids in the region between amino acids 250 to 595, only mutant 24 was found to bind DNA (lane 14), which localized the DNA-binding region of pp89 to amino acids 438 to 534. The TrpE fusion protein of this region did bind DNA with an affinity comparable to that of pp89 and mutants A, N and F. No reactivity was seen with the short fragments J, 5', H2B [containing the region homologous to histone H2B (Keil et al., 1987a)], 2 and 15 (lanes 8 to 13), which is in agreement with the results obtained using the deletion mutants. Similarly, the C-terminal fragments contained in A' and 3' did not react (lanes 15 and 16). The Coomassie blue-stained control gel shows that some lanes containing extracts of bacteria that should express these fragments contain less protein. However, when taking the 170K bacterial DNA-binding protein as
the internal standard, even longer exposures of the Southwestern blot, which revealed the reactivity of this protein, did not demonstrate the reactivity of any other fragment except 24 (lane 14). The DNA-binding capacity of pp89 fragments correlated strongly with the recognition of fragments by MAb 6/20/1 (Keil et al., 1985). This antibody recognizes the amino acid sequence between positions 468 and 492 (Koszinowski et al., 1987). Although this also demonstrates that this region is involved in DNA binding, there remains a certain ambiguity with respect to some of the smaller fragments that did not react. This is due to the fact that in the absence of a specific antiserum that reacts with all individual fragments the relative amount of the fusion proteins in bacterial lysates could not be determined accurately by Western blotting.

The HCMV IE1 protein does not interact with DNA

The immediate early 1 genes of both HCMV (Stenberg et al., 1984) and MCMV (Keil et al., 1987a) share a similar structure in that both contain four exons, exon 2 starts with the first ATG and exon 4 encodes the major component of the mRNA including the DNA-binding domain. No significant homologies between the nucleotide sequences exist and comparison of the amino acid sequences revealed only one region with similarity, within the glutamic acid-rich regions of both proteins (Keil et al., 1987a). Since the analysis of the DNA-binding activity of pp89 deletion mutants localized the DNA-binding domain within the highly acidic region of pp89, amino acids 424 to 532, a similar DNA-binding property of HCMV IE1 seemed plausible. To compare directly the DNA-binding properties of both proteins, extracts were run in parallel on the same gel, blotted onto nitrocellulose and incubated with labelled DNA (Fig. 5a). After autoradiography the nitrocellulose was dissected and proteins were analysed with either MCMV- or HCMV-specific sera (Fig. 5b). DNA was bound efficiently by the 145K β-gal–pp89 fusion protein (Fig. 5a, lane 2), whereas no DNA-binding activity of the

Fig. 4. DNA binding of pp89 deletion mutants. (a) Extracts of bacterial cells expressing β-gal (lane 1), β-gal–pp89 (lane 2), deletion mutants A, N, F, C, H and J fused to β-gal (lanes 3 to 8), TrpE (lane 9), or short sequences of pp89 5', H2B, 2, 15, 24, A' and 3' fused to TrpE (lanes 10 to 16) were separated by SDS–PAGE and Coomassie blue-stained. Arrowheads indicate fusion proteins identified by immunostaining with MAb 6/20/1 or with antisera raised against peptides of pp89. (b) Extracts of bacterial cells were separated by SDS–PAGE, transferred to nitrocellulose and incubated with a mixture of MCMV ss- and dsDNA 32P-labelled by random oligonucleotide-primed synthesis. The designation of lanes is the same as those in (a). Positions of pp89-specific DNA-binding proteins of the authentic size are marked by arrows. (c) pp89 domains expressed in the fusion proteins and their DNA-binding activity.
Fig. 5. DNA binding of the HCMV IE1 protein. (a) Extracts of bacterial cells expressing β-gal (lanes 1 and 3), β-gal–pp89 (lane 2) and β-gal–HCMV IE1 (lane 4) were analysed for DNA-binding proteins as described in Fig. 4. (b) After autoradiography, Western blotting with MCMV-specific (lanes 1 and 2) or HCMV-specific serum (lanes 3 and 4) was carried out.

110K β-gal–HCMV IE1 protein was observed (lane 4). Western blotting (Fig. 5b, lane 4) demonstrated that the HCMV IE1 protein was present on the nitrocellulose in this experiment and Coomassie blue staining revealed that the amount of the HCMV IE1 protein considerably exceeded that of pp89 (data not shown). Therefore we concluded that the HCMV IE1 protein differs from the MCMV IE1 protein pp89 with respect to DNA binding.

Discussion

pp89 is the prominent viral protein of the IE phase of MCMV infection. It can activate cellular genes (Schicke-danz et al., 1988) as well as heterologous viral promoters (Koszinowski et al., 1986), and contributes to the activation of the MCMV e1 gene by the ie3 gene product. In previous studies we have been able to show that IE proteins extracted from infected cells are not retained on DNA–cellulose columns unless histones are present.

In this report we show that a direct DNA-binding capacity of pp89 can be detected after separation of proteins by SDS–PAGE. Analysis of deletion mutants localized the DNA-binding domain within the region between amino acids 438 and 534. The capacity of pp89 to interact directly with DNA was not revealed by DNA–cellulose chromatography of cell extracts. We suggest that the failure of native pp89 to interact directly with DNA results from either a configuration that masks the DNA-binding region or that the DNA-binding region is blocked by the interaction of pp89 with other proteins. A similar feature has also been described for the NF-κB transcription factor. The DNA-binding site of this protein has been found to be blocked in cells that do not express κ-immunoglobulin light chain genes. The DNA-binding property could be activated in vitro by a denaturation-renaturation protocol, and the authors assumed that the DNA-binding site is inactivated in these cells by the interaction of NF-κB with an inhibitor (Baeuerle & Baltimore, 1988). The technique of South-western blotting for the detection of DNA-binding properties of cellular and viral proteins in combination with the expression of the proteins under study in the form of bacterial fusion proteins has turned out to be appropriate for the characterization of functional domains of proteins (Lenz et al., 1990; Moreland et al., 1991; Tsujimoto et al., 1991), including herpesvirus nuclear proteins (Inoue et al., 1991). For pp89 we could show that in this assay system the binding activity of pp89 and of cellular histones is comparable. Furthermore, the binding properties were not affected by the expression of pp89 as a bacterial fusion protein.

The MCMV ie1 gene product binds ss- and dsDNA, but shows a preference for ssDNA. Binding of specific DNA sequences could not be detected. This property is shared by a group of proteins such as GP5 from phage M13, GP32 from phage T4, and RecA, SSB and SSF from E. coli. All these proteins bind strongly and cooperatively to ssDNA without any apparent sequence specificity. They also share the ability to reduce the melting temperature of dsDNA by preferentially binding to ssDNA and preventing it from renaturing. Alignment of the DNA-binding regions of these proteins revealed a ssDNA-binding triple-stranded β-sheet motif and led to the hypothesis that the protein–ssDNA complex is stabilized by the interaction of aromatic and basic residues with the bases and phosphate groups, respectively, of the DNA (Venkataram Prasad & Chiu, 1987). The herpes simplex virus type 1 protein ICP8 also preferentially binds ssDNA, and its DNA-binding region has similarities with this motif (Wang & Hall, 1990).

Sequence analysis of the domain of pp89 with DNA-binding properties did not reveal a sequence homologous to this ssDNA-binding motif. The DNA-binding domain (amino acids 438 to 534) of MCMV pp89 is almost identical to the highly acidic domain spanning amino acids 424 to 532, which contains 42 acidic amino acids and only one basic amino acid. Sequence comparison of this region with known ds- and ssDNA-binding proteins did not show any significant similarities. This failure to detect homologies is not surprising because the majority of DNA-binding proteins studied interact with DNA via basic regions.

The HCMV IE1 protein also contains an extended C-
terminal acidic region, in which 36 of 103 amino acids are acidic. Therefore, similar DNA-binding capacities could be expected. However, the HCMV IE1 protein did not interact with DNA when tested in the same assay as pp89. On the other hand, an interaction between metaphase chromosomes and the HCMV IE1 protein has been described, and the acidic region was hypothesized to mediate this interaction (LaFemina et al., 1989). Therefore, the acidic region of each protein probably exerts a different function.

The isolated acidic region of pp89 still interacts with DNA when expressed as a TrpE fusion protein. Therefore we propose that it is a feature of this sequence itself which is responsible for the DNA-binding capacity and that adjacent amino acid sequences do not essentially contribute to the DNA-binding property. Acidic regions have been identified in several chromatin and chromosomal proteins, and are thought to play a role in unfolding condensed chromatin and in regulation of transcription by interacting with histones or transcription factors (Earnshaw, 1987). pp89 also interacts avidly with histones, but via a region adjacent to the acidic domain located between amino acids 71 and 415 (Münch et al., 1991). Therefore we speculate that pp89–histone complexes may interact with DNA via the DNA-binding Ser-Pro-Lys-Lys regions of histones (Suzuki, 1989) and the acidic region of pp89. However, the experimental approach to solve this question would be difficult, as both protein species interact with DNA in a sequence-independent manner.

Little is known about direct DNA interactions of protein acidic regions. The mammalian high mobility group proteins 1 and 2 (HMG1 and HMG2), which contain highly acidic regions of about 50 amino acids at the C terminus, bind to ssDNA, but the DNA-binding region of HMG1 has been localized to an N-terminal basic region (Reeves & Nissen, 1990). It has been suggested that the acidic region of HMGs is involved in denaturation of dsDNA because isolated polyglutamic acid regions have been shown to unwind DNA (Yoshida, 1983). pp89 binds ss- and dsDNA but shows a preference for ssDNA and thus we propose that the interaction of pp89 with dsDNA could result in unwinding of dsDNA, probably caused by interaction of acidic amino acids with the bases of the DNA. Experiments to study this question are under way.

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References


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