A *cis* Downstream Element Participates in Regulation of In Vitro Transcription Initiation from the P38 Promoter of Minute Virus of Mice

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We report the use of a HeLa whole cell extract (WCE) runoff transcription system for the study of *cis*- and *trans*-acting elements, participating in the regulation of transcription initiation from the P38 promoter of the parvovirus minute virus of mice (MVM). Our initial studies with HeLa WCE indicated that transcription from the P38 promoter is very inefficient, compared with transcription from the P4 promoter. Supplementation of the HeLa WCE with WCE prepared from uninfected Ehrlich ascites cells enhanced transcription from the P38 promoter twofold, indicating a role for a cellular factor in transcription from the P38 promoter. Furthermore, supplementation with WCE prepared from MVM-infected Ehrlich ascites cells enhanced transcription from the P38 promoter about sixfold, indicating a role for a virally encoded or induced factor. Analyses of runoffs produced by transcription of DNA templates digested with various restriction enzymes defined a downstream promoter element (DPE) necessary for efficient transcription initiation from the P38 promoter. This element resides 282 to 647 base pairs 3' to the transcription initiation site, between the *Nar*I site and the *Hind*III site (2287 to 2652, MVM numbering system). The virally encoded NS1 protein was shown by DNA precipitation to bind directly or indirectly through a cellular factor to the DPE. This interaction is suggested to be involved in the up regulation of the P38 promoter of MVM. Finally, with a DNase I protection assay performed on a fragment containing the DPE, we estimated the sequence involved in the binding of a factor present in uninfected and infected extracts. The correlation between the binding and transcription activation is discussed.

The role of promoter elements which reside 3' to a transcription initiation site (downstream promoter elements [DPE]) has been implicated in the control of gene expression in several cellular and viral genes. These include the α and β globin promoters (7, 33), the *Drosophila copia* transposable element (30), the rat somatic cytochrome *c* gene (15), immunodeficiency virus genes (19, 25), the adenovirus type 2 major late promoter (9, 20, 23), and the parvovirus H-1 (28).

Minute virus of mice (MVM) is a linear single-stranded DNA parvovirus containing 5,149 nucleotides (nt). It is an autonomous virus but is dependent upon cellular functions that are expressed during S phase for replication (6, 13, 14, 31). Transcription initiation catalyzed by the host RNA polymerase II has been shown to map to two promoters located at 4 (P4) and 38 (P38) map units (26). The two promoters have been more precisely mapped to nt 201 $\pm$ 5 and nt 2005 $\pm$ 4, respectively (4). Promoter P4 directs the transcription of two viral RNAs that are translated into the two nonstructural proteins, NS1 and NS2, while promoter P38 directs the transcription of the RNAs that are translated into the capsid proteins, VP1, VP2, and VP3 (10, 12, 13). It was suggested that, in the related H-1 parvovirus, two *cis* elements are active in the control of the P-38 promoter (28, 29). The first element (TAR) was found to be located upstream from the transcription initiation site and was shown to be active through an interaction with NS1 (29). The second *cis* element was found to be located up to 600 base pairs downstream from the transcription initiation site. Its action was not shown to involve NS1 (28). We decided to investigate the elements that are involved in controlling transcription initiation from the P38 promoter of MVM by using an in vitro approach. It has been shown in several

cases that whole cell extracts (WCEs) prepared from virus-infected cells, that are not themselves transcriptionally competent, can be added to a HeLa WCE that is transcriptionally competent to provide virally encoded or induced factors in *trans*, which are able to act upon an added DNA template in a manner similar to the in vivo situation (1, 21).

In this communication, we report the use of a HeLa WCE transcription system supplemented with WCEs prepared from uninfected and MVM-infected cells for the study of *cis* and *trans* elements involved in the transcriptional expression from the MVM P38 promoter. We show that the addition of uninfected or infected extracts enhances transcription from the P38 promoter about two- and sixfold, respectively, and that this activation is dependent upon a *cis* element which is located 3' to the transcription initiation site. This DPE is contained within 365 base pairs and is located between nt 2287 and 2652 of the MVM genome. The nonstructural protein NS1, was shown by DNA immunoprecipitation to bind directly or indirectly through a cellular factor to the DPE, and we suggest that this interaction is involved in the up regulation of transcription from the P38 promoter of MVM. Finally, by using a DNase I protection assay performed on a fragment containing the DPE, we estimated the sequence involved in the binding of a factor present in uninfected and infected extracts.

MATERIALS AND METHODS

Cells, viruses, and cell extracts. Ehrlich ascites (EA) or HeLa cells were grown in suspension in Dulbecco modified Eagle medium supplemented with 5% calf serum. For viral infections, one liter of EA cells at 1×10^5 to 2×10^5 cells per ml were collected by centrifugation and suspended in 250 ml of an MVM (T) stock virus (5). The virus was allowed to absorb for 2 h, and then the culture was diluted back to 1 liter

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with Dulbecco modified Eagle medium with 5% calf serum. Cells were harvested at 24 h postinfection. WCEs were prepared from infected and uninfected EA cells and HeLa cells as described (22).

Antibodies. Anti-NS1 sera were raised against a truncated NS1 protein encoded by nts 1110 to 1638 (given to us by P. Tattersall and S. Cotmore).

Chemicals. Nucleoside triphosphates and creatine phosphate were purchased from Sigma Chemical Co., St. Louis, Mo. [α - ^{32} P]UTP (400 Ci/mmol) was purchased from Amersham International, United Kingdom. [α - ^{32} P]dCTP (3,000 Ci/mmol) was purchased from Dupont, NEN Research Products, Boston, Mass.

DNA templates used for in vitro transcription. OCC-1 plasmid was constructed by insertion of a *Pst*I-*Pst*I MVM DNA fragment (1 to 416, MVM numbering) and an *Eco*RI-*Eco*RI MVM DNA fragment (1085 to 3158, MVM numbering) at the *Pst*I and *Eco*RI sites in the polylinker of the pGEM1 vector, respectively. OCC-2 plasmid was constructed by insertion of a *Pst*I-*Nco*I MVM DNA fragment (1 to 260, MVM numbering) and an *Eco*RI-*Eco*RI MVM DNA fragment (1085 to 3518, MVM numbering) at the *Pst*I and the *Eco*RI sites in the polylinker of the pGEM1 vector, respectively. In both constructs, the fragments were inserted so that transcription from both promoters proceeded in the rightward direction (see Fig. 1C and 2C for a schematic diagram of the plasmids).

DNA fragment used for immunoprecipitation of DNA with antisera and DNase I protection assays. The *Nar*I-*Hind*III 365-base-pair DNA fragment (2287 to 2652, MVM numbering) was purified from an agarose gel and end labeled by a filling-in reaction with Klenow fragment and [α - ^{32} P]dCTP. The fragment was designated H.

In vitro transcription reactions with WCE. Ten microliters of WCE was incubated for 15 min at 30°C with 0.5 to 1 μ g of DNA and 4 mM creatine phosphate in a final volume of 15 μ l (preinitiation step). WCEs used were either HeLa or HeLa supplemented with infected EA WCE (8:2) or HeLa supplemented with uninfected EA WCE (8.5:1.5). The optimal ratio of HeLa to supplementing extract was determined by titration of different amounts of extract against a fixed concentration of DNA. ATP, GTP, and CTP (final concentration, 500 μ M), UTP (final concentration, 10 μ M), and 20 μ Ci of [α - ^{32} P]UTP in 5 μ l were added, and the reaction mixture was incubated for 45 to 60 min at 30°C. In the case of pulse-chase reactions, after the preinitiation step, ATP, GTP, and CTP (final concentration, 500 μ M) and 30 μ Ci of [α - ^{32} P]UTP in 5 μ l were added and incubated for a 1-min pulse at 30°C, which was followed by the addition of 1 μ l of unlabeled UTP (final concentration, 500 μ M), and the reactions were allowed to proceed for another 30 min at 30°C. Reactions were stopped by the addition of 20 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 μ g of proteinase K per ml, and 30 μ g of tRNA and were incubated at 37°C for 15 min. RNA was phenol-chloroform extracted, purified by passage through a Sephadex G-25 minicolumn, and collected by ethanol precipitation. RNA was suspended in 10 μ l of 90% formamide and analyzed on 4% polyacrylamide gels (bisacrylamide, 1:19) containing 7 M urea (Schwartz/Mann, Orangeburg, N.Y.) and TBE (89 mM Tris borate [pH 8.3], 89 mM boric acid, 2 mM EDTA). Electrophoresis was carried out at a constant current of 20 mA.

Western blots and immunoprobings. Proteins from WCEs were boiled for 5 min in Laemmli sample buffer (6% SDS, 10% β -mercapthoethanol, 20% glycerol, 125 mM Tris hydrochloride [pH 6.8], and bromophenol blue) and analyzed on

10% acrylamide-bisacrylamide (30:0.8) gels, containing 0.4 M Tris hydrochloride (pH 8.8), 0.1% SDS (with a stacking gel containing 5% acrylamide), 0.1 M Tris hydrochloride (pH 6.8) and 0.1% SDS. Electrophoresis was carried out in Tris glycine buffer. Gels were then electroblotted for 2 h at 50 to 60 V in transfer buffer (25 mM Tris hydrochloride, 192 mM glycine, and 20% [vol/vol] methanol at pH 8.3) onto nitrocellulose filters (pore size, 0.2 μ m) as previously described (32). Blots were then prehybridized for 16 h at 4°C with phosphate-buffered saline, pH 7.0, containing 10% lowfat milk and 0.1% Tween. For hybridization, antisera were added to the same buffer and the filters were incubated for 2 h at 37°C. The filters were washed, and ^{125} I-labeled protein A (Amersham) was added and incubated for 2 h at 37°C. The filters were then washed and exposed to Agfa X-ray film.

Immunoprecipitation of DNA. Three microliters of antisera, binding buffer (40 mM Tris hydrochloride [pH 7.9], 0.1 M KCl, 10 mM MgCl₂, 0.2 mM EDTA, 2 mM dithiothreitol, 15% glycerol), 2 μ l of WCEs, and 1 μ g of poly(dI-dC) were incubated for 15 min at 30°C. End-labeled DNA was added (10⁴ cpm) and incubated for another 15 min at 30°C. Sepharose-protein A (3 mg) was added to each reaction in binding buffer, and reactions were allowed to shake for 1 h at 4°C. The reactions were washed in immunoprecipitation wash buffer (50 mM Tris hydrochloride [pH 7.5], 140 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.1% aprotinin) by centrifugation until there was no radioactivity in the supernatant. The first supernatants and the pellets were phenol-chloroform extracted and ethanol precipitated, and the DNA pellets were dissolved in sequencing gel sample buffer and analyzed on a 4% denaturing gel.

DNase I protection assay. End-labeled H DNA fragment (5 $\times$ 10⁴ cpm, 20 ng) was incubated for 20 min at 25°C with 15 μ l of WCE (100 μ g of protein) in binding buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 8.0], 0.1 mM EDTA, 100 mM NaCl). CaCl₂ was then added to a final concentration of 3.5 mM. DNase I (Worthington Diagnostics, Freehold, N.J.) was added to a final concentration of 0.08 U per reaction. Reactions were stopped after 1.5 min at 25°C. DNA was phenol-chloroform extracted, collected by ethanol precipitation, and analyzed on a 4% denaturing gel as described (16). G reaction (data not shown) was done as described previously (24).

RESULTS

In vitro transcription from the MVM P38 promoter is regulated by a trans-acting factor contained in uninfected and MVM-infected WCEs. To investigate the elements participating in the regulation of transcription from the P38 promoter, a construct was prepared that contained the two viral promoters P4 and P38. In this construct, the two promoters were brought closer together than they exist in the MVM genome in order to allow analysis of the transcripts from the two promoters on the same polyacrylamide gel (Fig. 1C).

Transcription of this template digested with *Bgl*I in HeLa WCE gave rise to two transcripts of 876 and 1,879 nt (Fig. 1A, HeLa lane). This is the expected size of transcripts originating from the P38 and P4 promoters, respectively (Fig. 1C). The ratio of the P38 runoff to the P4 runoff is lower than would have been expected in previous studies (26). After attempting to titrate several different plasmids against the HeLa WCE, we realized that higher levels of transcription from the P38 promoter could not be obtained in this system (data not shown). It was known from previous

FIG. 1. In vitro transcription with different WCEs. (A) The OCC-2 construct (detailed in Materials and Methods) cut with *Bgl*II was used as a DNA template for transcription. Lanes: a and b, the runoff transcripts obtained in reactions supplemented with WCE of infected EA cells containing 0.5 and 0.75 µg of template DNA, respectively; HeLa, the runoff transcripts obtained in a reaction containing HeLa WCE and 0.5 µg of DNA template; M, ³²P end-labeled ϕ X DNA digested with *Hae*III. (B) The template is the same as that described in 1A. Lanes: a and b, the runoff transcripts obtained in reactions supplemented with uninfected EA WCE containing 0.5 and 0.75 µg of template DNA, respectively; c and d, the runoff transcripts obtained in reactions supplemented with infected EA WCE, containing 0.5 and 0.75 µg of template DNA, respectively; α -amanitin, 0.5 µg of α -amanitin per ml added to a reaction identical to that described for lane d; HeLa, the runoff transcripts obtained in a reaction containing 0.75 µg of template DNA and HeLa WCE alone; M, size markers as described in 1A. (C) A scheme showing the MVM sequences (■) in the OCC-2 construct, the location of the P4 and P38 promoters, and the expected runoff lengths in nucleotides, obtained when the OCC-2 construct was cut with *Bgl*II.

studies that in vitro *trans* activation was possible by supplementation of HeLa WCE with extracts from other cells (described in Introduction and Discussion). We therefore tried to supplement the HeLa WCE with WCE prepared from MVM-infected EA cells, reasoning that a viral factor or a virally induced factor could be involved in *trans* activation of the P38 promoter. There was a strong enhancement of transcription from the P38 promoter when the HeLa WCE was supplemented with WCE from infected EA cells (24 h postinfection), while transcription from the P4 promoter was unaffected (Fig. 1A, lanes a and b). The effect of supplementation was most prominent when the ratio of HeLa WCE to WCE prepared from MVM-infected EA cells was 8:2. This was determined by calibrating one concentration of DNA against different mixtures of HeLa WCE and WCE from MVM-infected EA cells and choosing the ratio which best supported transcription from the P38 promoter (data not shown).

In order to verify whether the enhanced transcription from

the P38 promoter was due to the viral infection, the cell type, or both, the same experiment was done with WCE prepared from uninfected EA cells. Again, one concentration of DNA was calibrated against different mixtures of HeLa WCE and WCE prepared from uninfected EA cells. In this case, the ratio which best supported transcription from the P38 promoter was 8.5:1.5 (HeLa WCE to uninfected EA WCE) (data not shown). Although transcription from the P38 promoter was highest when the reaction was supplemented with WCE prepared from infected cells (Fig. 1B, lanes c and d), there was also an enhancement of transcription when the reaction was supplemented with WCE prepared from uninfected EA cells (Fig. 1B, lanes a and b, compared with the HeLa lane). All runoff transcripts in this experiment were shown to be α -amanitin sensitive, indicating that they are transcribed by RNA polymerase II (Fig. 1B, lane α -amanitin).

Identification of a *cis* element downstream from the P38 promoter that is involved in *trans* activation. In order to confirm that the transcripts designated to originate from the P38 and P4 promoters (Fig. 1) indeed initiated at their respective promoters, the template was digested with different restriction enzymes to cause a shift in the length of the runoff transcripts (Fig. 2C). When the template was digested with *Bgl*II, two transcripts, of 876 and 1,879 nt, were observed (Fig. 2C). When the template was cut with *Nar*I, the P4 transcript shifted to the expected length of 1,446 nt, while the transcript from the P38 promoter that was expected to be 283 nt was not visible. These reactions were performed under pulse-chase conditions so that only the 5' end region of the transcript would be labeled, making the amount of radioactivity incorporated independent of the length of the transcript. The reactions were performed in HeLa WCE mixed with infected EA WCE so the maximal transactivation effect was expected. The two bands of about 600 nt are α -amanitin insensitive and are therefore not RNA polymerase II transcripts (data not shown). The OCC-1 plasmid is identical to the OCC-2 plasmid except that sequences 260 to 416 (MVM numbering) were included following the P4 promoter. We have seen no differences in transcription patterns from the OCC-2 plasmid, compared with those from the OCC-1 plasmid, except the expected shift of runoffs from the P4 promoter (data not shown).

The fact that no P38 transcript could be observed when the template was cut with *Nar*I suggested that sequences downstream from the *Nar*I site may be important in *trans* activation of transcription from the P38 promoter. In order to investigate this possibility, the template was digested with *Hind*III, an enzyme that cuts between the *Nar*I site and the *Bgl*II site after the P38 promoter (Fig. 2C, for details of the expected runoff size). The template digested with *Hind*III supports transcription from the P38 promoter, and this transcription is dependent upon supplementation of the HeLa WCE with infected EA WCE (Fig. 2B; compare the ratio of the P4 and P38 runoffs between the HeLa and EA infected lanes). The transcripts are α -amanitin sensitive (data not shown). No transcripts were visible when no DNA was included in the reaction (Fig. 2B, lane No DNA).

The shift in the lengths of the transcripts from the P4 and P38 promoters when the template is cut with different restriction enzymes confirms that the observed transcripts do originate from the two MVM promoters. The results of this experiment suggest that the sequences between the *Nar*I site and the *Hind*III site are necessary for *trans* activation of the P38 promoter. When further narrowing of this essential element was tried, no conclusive results could be obtained

FIG. 2. Narrowing down the downstream sequences essential for P38 transcription. (A) Electrophoresis of transcription of HeLa WCE supplemented with infected EA WCE, under pulse-chase labeling conditions. Lanes: *NarI* (occ-1), the runoffs obtained when 0.5 µg of OCC-1 (cut with *NarI*) was transcribed in HeLa WCE supplemented with infected EA WCE; *BglI* (occ-2), the runoff transcripts obtained when 0.5 µg of OCC-2 DNA (cut with *BglI*) was transcribed in HeLa WCE supplemented with infected EA WCE (The P4 and P38 runoffs are indicated by closed and open arrows, respectively.); M, size markers as described in the legend to Fig. 1. (B) The OCC-2 DNA (0.75 µg) digested with *HindIII* was used as a template for transcription with HeLa WCE (lane HeLa) or HeLa WCE supplemented with infected EA WCE (lane EA infected). Lane No DNA is a control in which DNA was omitted from the transcription reactions. The P4 and P38 transcripts are indicated by arrows, as in 2A. Lane M shows size markers as described in the legend to Fig. 1. (C) A scheme showing the MVM sequences (■) 1 to 416 and 1085 to 3518 in the OCC-1 construct and 1 to 260 and 1085 to 3518 in the OCC-2 construct, the locations of the P4 and P38 promoters, and the expected runoff lengths, in nucleotides, obtained when OCC-1 and OCC-2 constructs are cut with *NarI*, *HindIII*, or *BglI*.

(data not shown), leaving the essential sequences for transcription from the P38 promoter to be defined as those between the *NarI* (residue 2288) and *HindIII* (residue 2655) sites.

The experiments described suggest that a viral product (perhaps in conjunction with a cellular factor present in the infected extract) may be responsible for the enhancement of transcription from the P38 promoter by binding to the DPE. One plausible viral product could be the NS1 protein (described in the introduction). In the H-1 parvovirus, NS1 was shown to have the ability to *trans* activate transcription from the P38 promoter *in vivo* (28, 29).

NS1 is present in infected EA cells. We checked to see whether NS1 protein is present in extracts prepared from infected EA cells and not in the other extracts used. Proteins

FIG. 3. Presence of NS1 in the different WCEs. Proteins from four extracts were analyzed on a 10% SDS-polyacrylamide gel and immunoblotted with anti-NS1 serum. Lanes: 1 and 2, infected EA (EA_{INF}) WCE; 3 and 4, uninfected EA WCE; 5 and 6, HeLa WCE; 7 and 8, infected EA WCE from a second preparation; M, molecular weight markers.

from four extracts—two infected EA extracts, a HeLa extract, and an uninfected EA extract—were run on 10% polyacrylamide-SDS gel, immunoblotted with anti-NS1 serum, and probed with ¹²⁵I-labeled protein A. The two infected extracts contain the 83-kilodalton NS1 protein (Fig. 3, lanes 1, 2, 7, and 8), while extracts from uninfected cells (lanes 3 and 4) and HeLa cells (lanes 5 and 6) do not contain any detectable NS1. The lower bands seen in Fig. 3, lanes 7 and 8, are probably due to some degradation of the protein or alternatively to modified forms of the NS1 protein (11).

NS1 binds directly or indirectly to sequences between the *NarI* and *HindIII* sites. We investigated whether NS-1 binds to the sequences after the P38 promoter that we found to be essential for up regulation of transcription from the P38 promoter. For this purpose, two assays were carried out: DNA immunoprecipitation and DNase protection. In both assays, we performed preliminary experiments to titrate the amount of WCE and the optimal conditions were used and shown.

To test the specificity of the reaction among NS1, anti-NS1 serum, and the H DNA fragment, we used DNA immunoprecipitation in the presence of control GEM-1 DNA fragments and a control antiserum. This type of assay has been discussed by Auborn et al. (3). Each reaction mixture contained the MVM H DNA fragment, extract from either infected (Fig. 4A) or uninfected (Fig. 4B) cells, and two GEM-1 DNA fragments. Binding was allowed to proceed for 10 min, and then each reaction was immunoprecipitated by either anti-NS1 or anti-*Drosophila* DER protein serum, which is not expected to recognize any of the WCE components, as a control. DNA was extracted from the pellets and supernatants and analyzed by polyacrylamide gel electrophoresis under denaturing conditions. The results, shown in Fig. 4A (pellet, lane αNS1), indicate that a significant fraction of the radioactivity associated with the H DNA fragment was precipitated with the anti-NS1 serum. The remainder of the radioactivity associated with the H DNA fragment was retained in the supernatant as were most of the

FIG. 4. Immunoprecipitation of DNA. (A) End-labeled DNA fragment H was incubated with end-labeled GEM-1 DNA fragments and WCE from infected EA cells. Three microliters of either anti-NS1 (α NS1) or anti-D (α D) serum (anti-*Drosophila* DER protein) was added, and the reactions were precipitated. DNA was extracted from the pellets and supernatants and was analyzed on a 4% polyacrylamide gel. The H DNA fragment and the GEM-1 DNA fragments are indicated by arrows. (B) The reaction was as described in 4A except that WCE from uninfected EA cells was included instead of WCE from infected EA cells.

control GEM-1 DNA fragments (Fig. 4A, supernatant, lane α NS1). The control reaction with the nonspecific antiserum (Fig. 4A, lanes α D) showed a slight precipitation of the MVM H DNA fragment, but it also precipitated the GEM-1 DNA fragments with the same efficiency as that seen in a longer exposure of the same gel (data not shown), indicating a nonspecific precipitation of all DNA fragments (Fig. 4A, pellet, lane α D). In Fig. 4, panel B, we show that when WCE from uninfected EA cells is included as a control, there is no preferable precipitation of the H DNA fragment. We therefore conclude that the MVM DNA fragment H is specifically precipitated by anti-NS1 serum in the presence of infected extract.

A cellular factor binds to the sequences between the *Nar*I and *Hind*III sites. In order to check whether there is a cellular factor capable of binding to the H DNA fragment, we compared DNase I protection patterns of the end-labeled H DNA fragment incubated in HeLa WCE, infected EA WCE, or uninfected EA WCE (Fig. 5). A comparison of these digestion patterns shows that there is a specific region (indicated by arrows and solid brackets in Fig. 5) that is protected. The protection is the same whether HeLa WCE, infected EA WCE, or uninfected EA WCE was used (Fig. 5A). Titration of different amounts of the WCEs in the footprint assays has not indicated a significant difference in the amount of the cellular factor. However, we cannot exclude this possibility because of the sensitivity of the assay. In some cases, the region protected by the infected EA WCE is somewhat extended (indicated by the broken bracket in Fig. 5B). This could be due in part to an unstable association between NS1 and a cellular factor. From the pattern of the G reaction (data not shown) and the markers, it can be estimated that the protected region is contained between residues 2491 to 2548 (MVM numbering). The results suggest the presence of the same DNA-binding factor in all the extracts tested.

FIG. 5. Footprinting of the H DNA fragment. End-labeled DNA fragment H was incubated with HeLa cells, infected EA cells, uninfected EA WCE, or no WCE, partially digested with DNase I, and analyzed by polyacrylamide gel electrophoresis under denaturing conditions. The estimated protected region is indicated by the bracket, and the band which disappears in the presence of WCE is indicated by an arrow in both panels. The extended region protected with the infected EA WCE is indicated by the broken bracket. The sequence included in the estimated protected region is shown, and the stars represent the protected Gs. Lane M shows size markers as explained in the legend to Fig. 1.

DISCUSSION

The results presented in this communication describe the adaptation of an in vitro transcription system with HeLa WCE supplemented with WCEs from uninfected and MVM-infected EA cells to the study of the elements that constitute the P38 promoter of MVM. The present study reveals an up-regulating element residing 282 to 647 base pairs downstream from the transcription start site, designated DPE. Truncation of the template upstream of this element prevents the appearance of a runoff transcript from the P38 promoter. Transcription from the P38 promoter by using a template truncated downstream of this element is enhanced when an extract prepared from uninfected or infected EA cells is added to the HeLa WCE, indicating that a factor in EA cells, modified or induced by a viral factor, is involved in promoting transcription from the P38 promoter. From previous in vivo studies (28, 29) and from our present results, it is suggested that the viral product NS1 is responsible in part for this *trans* activation. In the present study, we have not discriminated between initiation of transcription and elongation, but earlier work (4) (in which in vivo-initiated viral transcriptional complexes were isolated from cells and truncated upstream of the DPE, identified herein, and elongated in vitro) showed an efficient transcription from the P38 promoter. These observations indicate that the DPE is

essential for transcription initiation but not elongation. Furthermore, the inefficient transcription from the P38 promoter is not due to promoter interference by transcripts originating from the P4 promoter because similar results were also obtained when a construct containing only the P38 promoter was transcribed *in vitro* (data not shown). In the related H-1 parvovirus, a DNA sequence designated TAR, situated upstream of the P38 promoter, was also suggested to be involved in *trans* activation of the P38 promoter (28, 29). From the present study, it is apparent that the TAR sequence is not sufficient to enhance transcription from the MVM P38 promoter *in vitro*. It is possible that TAR is active only in the presence of the downstream element or that it is not the actual *cis* element responsible for *trans* activation by NS1.

The observation from RNA and protein analysis during infection (8, 14), in which it was shown that the two MVM promoters are expressed in a temporal order leading first to the production of the NS proteins and only later to the expression of the genes encoded by the P38 transcription unit, supports the assumption that NS proteins are essential for expression from the P38 promoter. The DPE reported here is most responsive when HeLa WCE is supplemented with WCE from EA cells infected with MVM. We therefore analyzed the binding of NS1 from MVM-infected extracts to the DPE by immunoprecipitation. With this assay, we were able to show that NS1 binds directly or indirectly to the DPE. The footprint mapping of the DPE indicates that a cellular factor binds to the DPE. It is possible that NS1 binds to the DNA through an interaction with this cellular factor.

The binding of the cellular factor as assessed by DNase footprinting is similar with all three extracts tested, although supplementation of HeLa WCE with WCE from uninfected EA cells led to transcription above the basal level detected with HeLa WCE alone but not to the extent of enhancement seen with supplementation with WCE from MVM-infected cells. This could be explained by assuming that a cellular factor which binds to the DPE is present both in EA cells prior to infection and in HeLa cells but in a lower amount in the latter. This might be insignificant when binding is tested by footprinting but could be crucial when tested by a functional assay as *in vitro* transcription. An explanation which is not mutually exclusive could be that the cellular factor which binds to the DPE appears in two forms, one of which is modified. This modification does not affect binding but has an effect on transcription activation. In this case, the footprint analysis of the DPE would appear similar when HeLa WCE or WCE from uninfected or infected EA cells is used. On the other hand, when *trans* activation is tested in a transcription reaction, the modified factor from uninfected, or more so from infected, EA WCE will show activation above the basal level seen with HeLa WCE. We suggest that upon viral infection, this modification of a preexisting cellular factor is further enhanced. From studies carried out in other systems, it was shown that viral proteins such as E1A of adenovirus (18) and vmw65 of herpesvirus (17, 27) are able to modify a preexisting cellular component and thus improve its ability to *trans* activate transcription. From a sequence comparison reported (2), it was shown that NS1 has an ATPase activity domain and other characteristics which are also shared with proteins that are suggested to act by modification of other proteins and thereby *trans* activate transcription. Purification of the factors involved and direct addition to HeLa WCE will help to define their role in *trans* activation of the P38 promoter.

The significance of a promoter element residing down-

stream from the transcription start site could be explained by the mode of its action. It seems that this *cis* element is responsive to *trans* factors which are distinct from those interacting with ubiquitous promoter elements such as the TATA box, CCAAT motif, and SP1- and AP1-binding sequences. It could be postulated that this DPE, when bound to a factor or to a complex of factors, activates initiation by looping or changing the conformation of the template in a way which makes it more vulnerable to make contact with an upstream factor or the polymerase to form a committed initiation complex. The special localization of this control element, as far as promoters are known, might enable a finer and more complex regulation of transcription directed by the promoter during viral infection.

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