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Characterization of Adeno-Associated Virus Rep Protein Inhibition of Adenovirus *E2a* Gene Expression

Shadia Nada and James P. Trempe[1]

Department of Biochemistry and Molecular Biology, Medical College of Ohio, 3035 Arlington Avenue, Toledo, Ohio 43614-5804

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Adeno-associated virus (AAV) replication (Rep) proteins are pleiotropic effectors of viral DNA replication, RNA transcription, and site-specific integration into chromosome 19. In addition to regulating AAV gene expression, the Rep proteins modulate expression of a variety of cellular and viral genes. In this report we investigate Rep-mediated effects on expression of the adenovirus (Ad) *E2a* gene and the Ad major late promoter. We have found that all four Rep proteins repress *E2a* expression at the protein level, with Rep40 showing the weakest repression. Mutations in the purine nucleotide binding (PNB) site weakened each of the protein's abilities to repress expression. Analysis of steady-state *E2a* mRNA showed that Rep proteins decreased mRNA levels, but to a lesser extent than E2a protein levels. Analysis of mRNA stability demonstrated that neither Rep78 nor Rep52 affected *E2a* mRNA stability, suggesting that the decrease in mRNA is due to Rep-mediated inhibition of Ad *E2a* transcription. To determine if Rep68 proteins could directly inhibit RNA transcription, we performed *in vitro* transcription assays using HeLa nuclear extracts supplemented with Rep68 and Rep68PNB. We demonstrate that Rep68, but not mutant Rep68PNB, blocked *in vitro* transcription of a template containing the Ad major late promoter. These results provide insight into how AAV and its encoded Rep proteins interact with Ad and provide a model system for the study of AAV and host-cell interactions.

Key Words: AAV; Rep protein; Ad *E2a;* AdMLP *in vitro* transcription.

INTRODUCTION

Adeno-associated virus (AAV) is a defective human parvovirus that normally requires a helper virus to complete its replication cycle (reviewed in Muzyczka and Berns, 2001). A variety of DNA viruses provide essential helper functions for AAV replication but adenovirus (Ad) is its most efficient helper (Atchison *et al.,* 1965; Casto *et al.,* 1967a; Atchison, 1970; Dolin and Rabson 1973; Buller *et al.,* 1981; McPherson *et al.,* 1985; Walz *et al.,* 1997; Ogston *et al.,* 2000). One recent report suggests that AAV replicates autonomously in differentiating keratinocytes (Meyers *et al.,* 2000). AAV integration into the long arm of chromosome 19 occurs in the absence of a helper virus co-infection and is the means by which the virus establishes latency in the infected cell (Muzyczka and Berns, 2001).

The AAV replication, *rep,* gene encodes four pleiotropic nonstructural proteins. These proteins, Rep78, Rep68, Rep52, and Rep40, are referred to as Rep proteins because expression of the larger proteins is required for viral DNA replication. The Rep proteins also modulate viral gene expression, site-specific integration into the chromosome 19 locus, and cellular gene expression (Muzyczka and Berns, 2001). The Rep78 and Rep68 roles in viral gene expression include repression of transcription from the p5 promoter in the presence and absence of Ad co-infections (Beaton *et al.,* 1989; Kyostio *et al.,* 1995; Pereira *et al.,* 1997), activation of transcription from the p19 and p40 promoters in the presence of Ad co-infection (McCarty *et al.,* 1991; Pereira *et al.,* 1997), and suppression of protein translation of *cap* gene mRNA (Trempe and Carter, 1988). In plasmid cotransfection experiments, *rep* gene expression inhibits gene expression from some heterologous promoters (Antoni *et al.,* 1991; Horer *et al.,* 1995; Khleif *et al.,* 1991; Labow *et al.,* 1987) and increases expression from the *c-sis* gene (Wonderling and Owens, 1996) and the CMV early promoters (Wonderling *et al.,* 1997).

AAV and the other parvoviruses have long been known to inhibit oncogene-mediated cellular transformation and the proliferation of infected cells. Helper virus-free AAV infection alters expression of several cell cycle-regulated genes (Hermanns *et al.,* 1997), promotes differentiation (Klein-Bauerschmitt *et al.,* 1992; Winocour *et al.,* 1992), and induces a cell cycle block (Winocour *et al.,* 1988). The AAV-induced cell cycle block is correlated with a decrease in retinoblastoma (Rb) protein phosphorylation and an increase in $p21^{Cip}$ gene expression (Hermanns *et al.,* 1997). AAV inhibitory effects are also exerted on the helper virus in co-infected cells in that AAV, but not defective interfering particles, blocks Ad DNA replication (Carter *et al.,* 1979; Casto *et al.,* 1967a,b; Laughlin *et al.,*

[1] To whom correspondence and reprint requests should be addressed. Fax: (419) 383-6228. E-mail: jtrempe@mco.edu.

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1979). AAV blocked Ad DNA replication more effectively than ultraviolet light inactivated AAV or an AAV vector (Jing *et al.,* 2001). These combined results indicate that amplification of the AAV genome or AAV gene expression is essential for Ad inhibition. The AAV effects on Ad-induced tumorigenicity and cellular transformation have been attributed to the AAV terminal repeat sequences and AAV gene expression (de la Maza and Carter, 1981; Khleif *et al.,* 1991); however, the effects on Ad replication have been attributed to expression of the AAV replication (*rep*) gene (Weitzman *et al.,* 1996). A recent report suggests that AAV alters cellular E2F expression in uninfected and Ad-infected cells (Batchu *et al.,* 2001). It was also proposed that AAV Rep78 stabilizes the pRb–E2f complex and binds to DNA elements in the E2F transcription promoter. In another report, Rep78 arrested cells in S phase by a novel mechanism involving the ectopic accumulation of active pRb (Saudan *et al.,* 2000).

In order to further define the interactions between AAV and its helper virus, we have begun a study of the inhibitory effects of AAV on Ad replication and gene expression. We present evidence that the AAV Rep proteins inhibit Ad *E2a* gene expression at the protein and mRNA levels. We also provide evidence that the effects on *E2a* mRNA accumulation may be due to effects on transcription rather than on mRNA stability.

RESULTS AND DISCUSSION

AAV *rep* vectors and protein expression

Individual Rep vectors were constructed to insure that only one of the four Rep proteins was predominantly expressed in plasmid-transfected 293 cells. A Met → Gly change was introduced into the Rep78 and Rep68 plasmids at the initiation codon for the Rep52/40 proteins to create pCDMRep78G and pCDMRep68G, respectively. This change results in the expression of fully functional Rep78/68 proteins and no expression of Rep52/40 (Chejanovsky and Carter, 1989). A mutation was introduced into all four Rep plasmids to inactivate the purine nucleotide binding site (Chejanovsky and Carter, 1990). Figure 1 shows diagrams of the pCDMRep plasmids used in this study. Ad-infected 293 cells were transfected with the eight plasmids to verify Rep protein expression (Fig. 2). The Rep78/68 proteins were immunoprecipitated with Rep-specific antisera because a cross-reacting cellular protein of approximately 68 kDa is frequently detected in immunoblot analyses and comigrates with the Rep68 protein (Trempe *et al.,* 1987). The immunoprecipitated proteins were then analyzed by immunoblot analyses. The immunoblot in Fig. 2 shows that the purine nucleotide binding (PNB) site mutant versions of the Rep proteins were expressed at levels similar to those of the wild-type form of the protein.

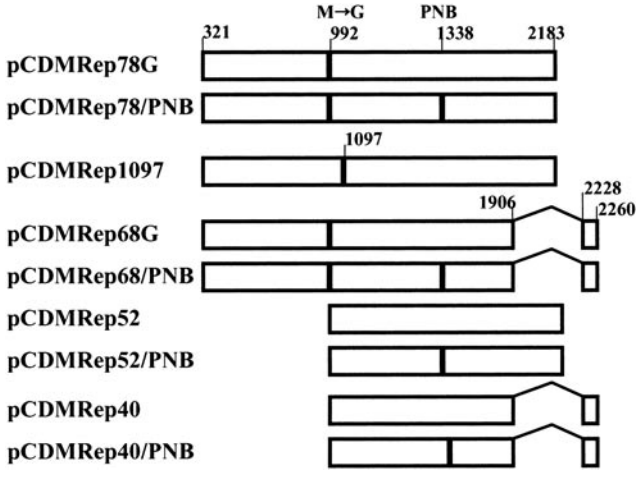

FIG. 1. Rep expression vectors. The drawing shows the individual Rep proteins expressed in cell culture from pCDM8-derived plasmids. The numbers are the locations in the AAV2 genome of the Rep78/68 initiation codon (321), the Met → Gly change in Rep78/68 (992), the mutation in the PNB site (1338), the Rep78/52 termination codon (2183), the major splice site (1906–2228), and the Rep68/40 termination codon (2260). The location of the *Xho*I linker in Rep1097 is indicated.

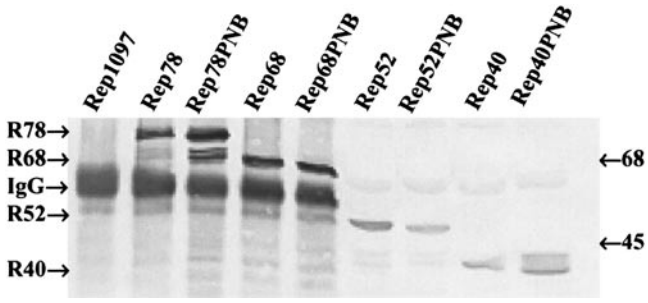

FIG. 2. Rep protein expression in 293 cells. The plasmids described in Fig. 1 were transfected into 293 cells. Rep proteins were immunoprecipitated with Rep-specific antisera (Trempe *et al.,* 1987), separated on SDS–PAGE, and immunoblotted with Rep antisera. The first five lanes containing the IgG band were the samples that were immunoprecipitated prior to gel electrophoresis. Alternatively cytoplasmic extracts were prepared, separated by SDS–PAGE, and analyzed by immunoblot. The locations of Rep proteins and IgG are indicated on the left. The locations of molecular weight standards are shown on the right.

AAV Rep protein effects on gene expression from the *E2a* transcription promoter

We have shown that Ad *E2a* expression is repressed in AAV and Ad co-infected HeLa cells and in a 293-derived cell line (Neo6) that inducibly expresses the AAV *rep* gene (Jing *et al.,* 2001). All four Rep proteins are expressed in a co-infection and the Rep78 and Rep52 proteins are expressed upon induction in Neo6 cells (Yang *et al.,* 1994). To determine if the individual Rep proteins affect expression from the *E2a* promoter, 293 cells were transfected with the Rep plasmids and a luciferase gene reporter under the control of the Ad *E2a* transcription promoter, p*E2a-luc.* Two days after trans-

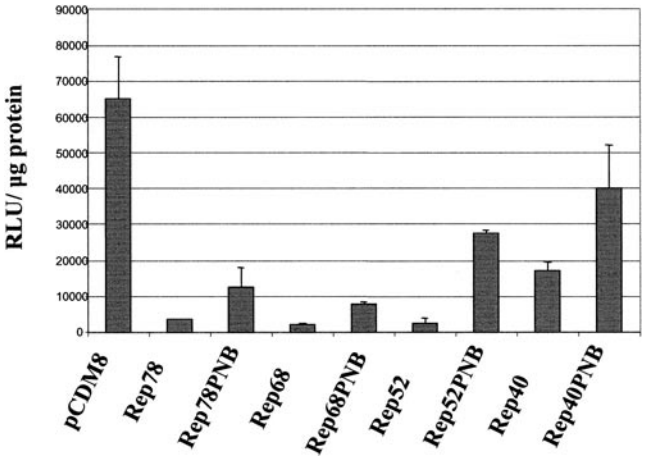

FIG. 3. AAV Rep protein effects on *E2a-luc* gene expression. The p*E2a-luc* plasmid was transfected into 293 cells with the indicated AAV Rep plasmids. 48 h later extracts were prepared and analyzed for luciferase activity. The results are reported as relative light units (RLU) per microgram of protein.

fection, cellular extracts were prepared and luciferase enzyme activity was determined. Figure 3 shows that all of the Rep-expressing plasmids suppress expression from the Ad *E2a* promoter and that Rep40 is the weakest suppressor. Each of the PNB mutant plasmids allowed higher activity than their corresponding wild-type plasmids. These results are consistent with other studies that have examined the ability of the individual Rep proteins to repress gene expression from homologous and heterologous promoters (Kyostio *et al.,* 1995; Horer *et al.,* 1995).

To determine if comparable inhibitory effects are realized with the complete Ad5 *E2a* gene, similar transfection experiments were performed using a plasmid containing a 5768-bp *Bam*HI–*Eco*RI DNA fragment from Ad5 that contains the entire *E2a* gene. 293 cells were cotransfected with the *E2a* and Rep-expressing plasmids and E2a protein expression was examined by immunoblot analysis (Fig. 4). Compared to the pCDM8 vector and pRep1097 null mutant, the Rep78, Rep68, and Rep52 proteins were the strongest suppressors of expression from the *E2a* promoter. The PNB mutant versions of the proteins were weaker suppressors than the wild type. In multiple repeats of this experiment, the Rep52 and Rep78 proteins were the strongest suppressors of E2a protein expression. These results are comparable to those obtained in the luciferase assays in that the PNB mutant versions of the Rep proteins were weaker suppressors of protein expression.

To determine if the diminished E2a protein expression is reflected in the steady-state *E2a* mRNA levels, 293 cells were transfected with the various Rep protein plasmids and the *E2a* gene plasmid, total RNA was isolated after 48 h, and mRNA levels were measured by Northern hybridization. Prior to the Northern analyses, E2a protein levels were examined by immunoblot and in all cases the levels of E2a were comparable to those observed in the immunoblot shown in Fig. 4 (data not shown). In the mRNA analysis, all of the Rep proteins except Rep68PNB and Rep40PNB reduced the level of *E2a* mRNA (Fig. 5). We consistently observed that Rep52, followed by Rep78, is the strongest suppressor of *E2a* mRNA levels as well as luciferase and E2a protein expression. In contrast, Horer *et al.* found that Rep78 and Rep68 suppressed mRNA levels from the HIV long terminal repeat and the HPV18 URR promoters, whereas Rep52 had only a modest effect in HeLa cells

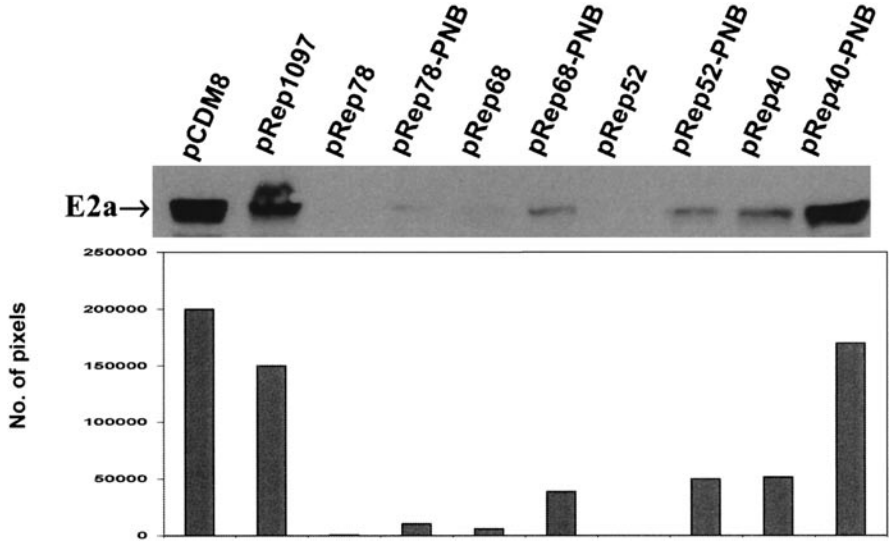

FIG. 4. AAV Rep protein effects on E2A protein expression. The pAd5E2a plasmid was cotransfected into 293 cells with the indicated AAV Rep plasmids. 48 h later the cultures were harvested and analyzed for E2A expression by SDS–PAGE and immunoblot using anti-E2ADBP antibody. The E2A band is shown in the immunoblot at the top. A densitometer scan of the immunoblot is shown at the bottom.

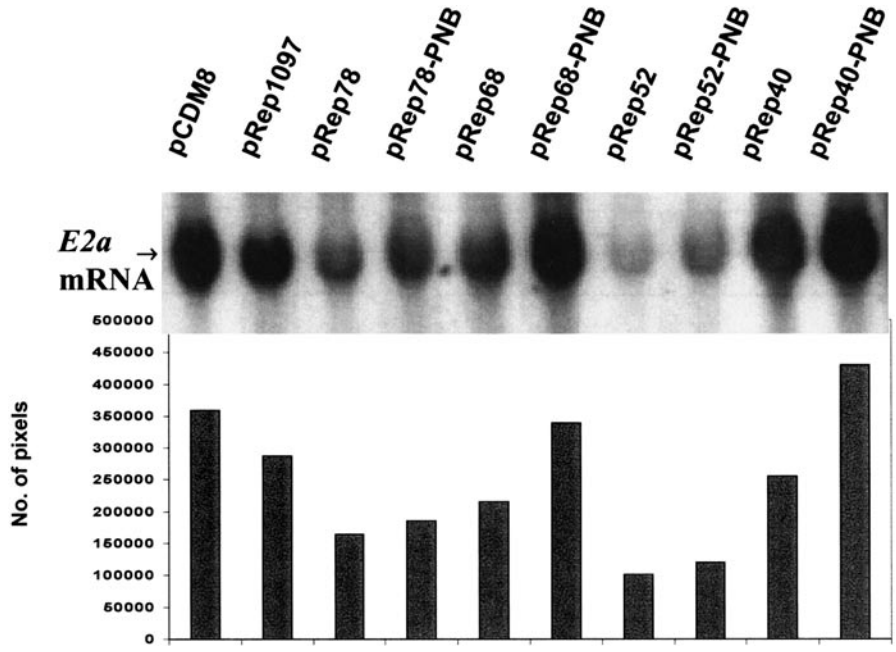

FIG. 5. AAV Rep protein effects on *E2a* mRNA levels. The pAd5E2a plasmid was cotransfected onto 293 cells with the indicated AAV Rep plasmids. 48 h later the cultures were harvested and *E2a* mRNA levels were analyzed by Northern hybridization. The *E2a* mRNA band is shown in the Northern blot at the top. A densitometer scan of the Northern blot is shown at the bottom.

(Horer *et al.,* 1995). The inability of Rep52 to site-specifically bind to DNA suggests that it may inhibit gene expression by a mechanism different from that of Rep78 or Rep68. Luciferase activity from the HIV and HPV promoters was repressed by Rep78 followed by Rep52, Rep68, and Rep40 (Horer *et al.,* 1995). Thus, Rep protein effects on mRNA levels and protein expression vary among transcription promoters and host cells.

It is interesting to note here that although Rep68PNB does not show any suppression of *E2a* mRNA, it suppressed expression of the E2a protein shown in Fig. 4 and luciferase expression from the *E2a* promoter shown in Fig. 3. These results suggest that Rep68PNB has lost the ability to suppress mRNA accumulation, and perhaps transcription, yet retains its ability to suppress mRNA translation. Thus Rep ATPase or helicase activity may not be required for inhibition of translation.

Ad5*E2a* mRNA stability

The quantity of any protein synthesized per unit of time depends on the abundance of its mRNA and the efficiency with which that mRNA is translated. In turn, mRNA abundance depends on the rate at which that mRNA is transcribed, spliced, processed, transported, and degraded (Peltz *et al.,* 1991). We performed an analysis of *E2a* mRNA stability to determine if the inhibition of *E2a* mRNA accumulation is due to effects on mRNA turnover rather than an effect on transcription. 293 cells were cotransfected with the Rep78, Rep52, and Ad5*E2a* plasmids. Forty hours posttransfection, actinomycin D was added to the cultures. At 2-h intervals up to 12 h, total RNA was isolated and analyzed by gel electrophoresis and Northern hybridization. Figure 6 contains a representative Northern hybridization and densitometer analysis of one such experiment. At time zero, *E2a* mRNA in the presence of Rep78 or Rep52 is 25–30% of the level of mRNA in cells cotransfected with the pCDM8 control vector. With increasing times of actinomycin exposure to block new transcription, there was a decrease in *E2a* mRNA level in all samples. The half-life of the mRNA in the pCDM8-transfected cultures is approximately 10 h, whereas in the Rep78- and Rep52-transfected cultures the half-life is approximately 11–12 h (Fig. 6C). Although actinomycin is known to have toxic effects on cells in culture (Peltz *et al.,* 1991) the lack of obvious degradation of the 28S and 18S rRNA (Fig. 6B) indicates that the total RNA integrity is not altered in these incubations. These results suggest that the stability of *E2a* mRNA is not altered by Rep78 or Rep52 and that the decrease in *E2a* mRNA is likely due to a Rep effect on transcription.

Rep protein effects on mutant Ad *E2a* promoters

Our results thus far suggest that the AAV Rep proteins decrease the steady-state level of Ad *E2a* mRNA but do not alter mRNA stability. This apparent Rep-mediated transcription effect may be regulated via specific transcription factor binding sites in the *E2a* promoter. To determine whether any of the known transcription factor binding sites in the *E2a* promoter are involved in suppression, we obtained a series of plasmids containing

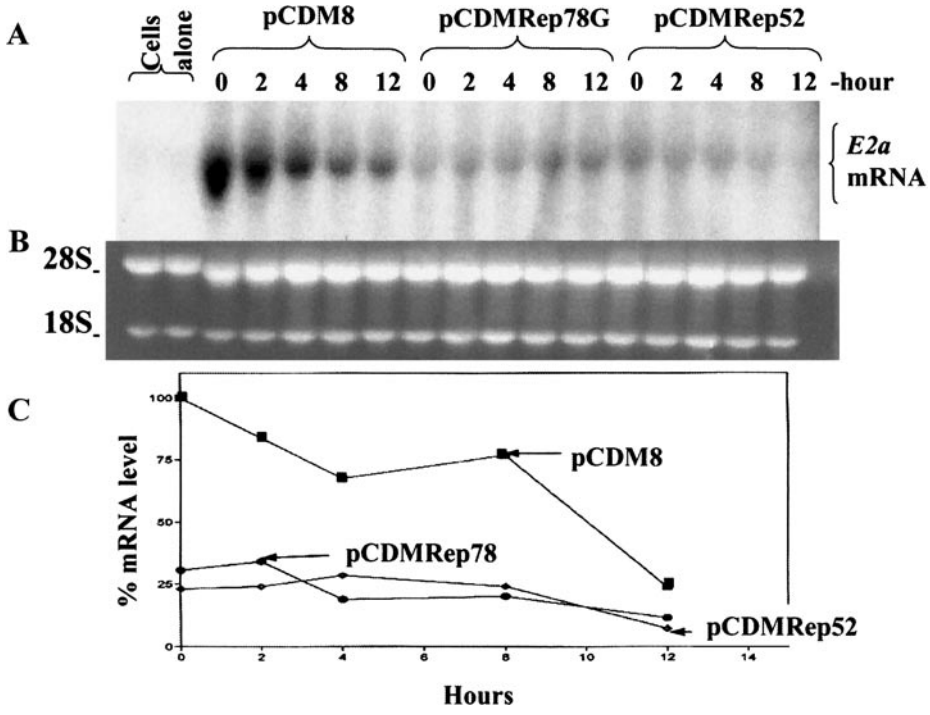

FIG. 6. AAV Rep protein effects on *E2a* mRNA stability. The pAd5E2a plasmid was cotransfected onto 293 cells with pCDM8, pCDMRep52, or pCDMRep78. 40 h posttransfection, actinomycin D was added to each dish at a concentration of 5 μg/ml. The cells were harvested 0, 2, 4, 8, and 12 h later and total RNA was isolated and analyzed by Northern hybridization. (A) Northern analyses showing the level of *E2a* mRNA at each of the indicated time points. (B) The gel was stained with ethidium bromide prior to RNA transfer to filter paper. The locations of the 18S and 28S rRNA species are indicated. (C) The relative levels of mRNA produced for the three cotransfections was determined by densitometer tracing of the Northern blot in A above. The amount of *E2a* mRNA at 0 h in the pCDM8 cotransfection was designated 100%. The amounts of mRNA in the other transfections are reported as a percentage of the pCDM8 cotransfection. These results are representative of three independent plasmid transfections.

wild-type and deletion mutant versions of the Ad *E2a* promoter attached to the chloramphenicol acetyltransferase reporter gene, *E2a-Cat* (Loeken and Brady, 1989). A diagram of the wild-type and deletion mutant promoter elements is shown in Fig. 7. Each of the *E2a-Cat* plasmids was cotransfected onto 293 cells in the presence of the Rep78, Rep68, or Rep52 plasmids. Forty-eight hours later cultures were harvested, cellular extracts were prepared, and Cat enzyme activity was determined. If the Rep proteins reduce expression by altering the ability of one of the transcription factors to stimulate promoter activity, then we would expect to see a change in relative Cat activity if that transcription factor binding site is removed from the promoter. The results in Table 1 are normalized to 100% for each of the four reporter plasmids. As expected, the wild-type plasmid expressed the most Cat activity followed by *E2ATF*$^-$ (38% of wt), *E2E2F*$^-$ (17% of wt), and *E2TATA*$^-$ (12% of wt) (results not shown). Deletion of the ATF, E2F, or TATA DNA elements did not result in an increase in gene expression in the presence of the wild-type Rep plasmids compared to the vector plasmid (pCDM8). Although reports in the literature suggest that Rep proteins may antagonize transcription pro-

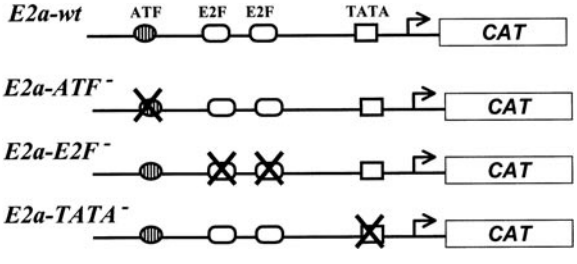

FIG. 7. Wild-type and mutant *E2a-Cat* plasmids. The wild-type *E2a* promoter attached to the *Escherichia coli Cat* gene is shown at the top. The ATF, E2F, and TATA deletion mutants are shown.

TABLE 1

Rep-Mediated Inhibition of Wild-Type and Mutant *E2a* Promoter Activity[a]

	E2a wt	*E2a ATF*$^-$	*E2a E2F*$^-$	*E2a TATA*$^-$
pCDM8	100	100	100	100
pCDMRep78	9.4 ± 4.6	13.4 ± 5.2	13.4 ± 4.0	10.2 ± 2.9
pCDMRep68	12.0 ± 4.3	19.9 ± 12.1	12.8 ± 3.9	19.0 ± 5.8
pCDMRep52	7.4 ± 0.3	14.9 ± 6.4	12.7 ± 1.1	10.7 ± 1.0

[a] 293 cell cultures were cotransfected with the various *E2a* promoter *Cat* plasmids and Rep plasmids. 48 h later cultures were harvested and Cat assays performed as described in the text. The values are reported as percentage of activity compared to the pCDM8 vector-transfected cultures ± standard deviation.

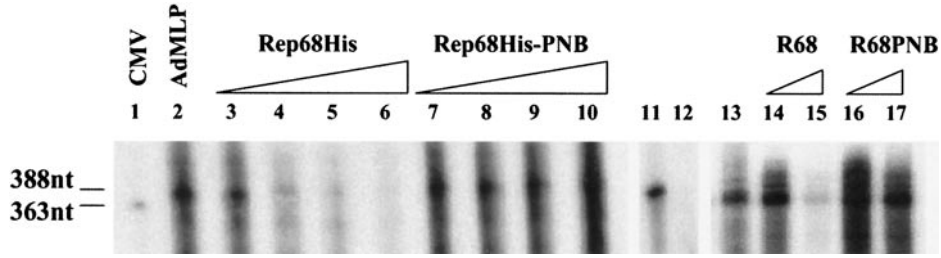

FIG. 8. Rep68 inhibits *in vitro* transcription of the Ad major late promoter. HeLa nuclear extracts were programmed for *in vitro* transcription using transcription templates driven by the cytomegalovirus early promoter (CMV) (lane 1) or the Ad major late promoter (AdMLP) (lane 2). The *Xba*I-linearized pTIGL template was used in lanes 2–12. The transcription reactions were supplemented with purified Rep68His (lanes 3–6) or Rep68His-PNB (lanes 7–10) at final concentrations of 0.032, 0.064, 0.096, and 0.128 μg/ml, respectively. Lanes 11 and 12 contain linearized AdMLP transcription in the absence and presence of 1 μg/ml of α-amanitin, respectively. Lanes 11–17 contain transcription products from circular pTIGL plasmid in the absence of added Rep68 (lane13) or in the presence of Rep68 (lanes 14, 15) or Rep68PNB (lanes 16, 17) at final concentrations of 0.064 (lanes 14, 16) and 0.128 μg/ml (lanes 15, 17), respectively. The locations of the control 363-nt CMV product and the 388-nt AdMLP products are indicated at the left.

moters containing sites for ATF (Chiorini *et al.,* 1998; DiPasquale *et al.,* 1998), TATA (Su *et al.,* 2000; Hermonat *et al.,* 1998), and E2F (Batchu *et al.,* 2001), it is clear that removal of only one of these binding sites from the *E2a* promoter is insufficient to alleviate Rep repression. Thus Rep-mediated inhibition of the Ad *E2a* promoter is not solely dependent upon one of these specific transcription factor binding sites.

Rep68 inhibits *in vitro* transcription of the Ad major late promoter

The diminution of *E2a* mRNA levels and the lack of an effect on mRNA stability strongly suggest that the Rep proteins inhibit transcription. However, the lack of an effect on expression from the *E2a* promoter mutants suggests that if the Rep proteins inhibit transcription, the target of Rep action may be other factors in the general transcription machinery or some other DNA element in the plasmid vector. To determine if purified Rep68 protein affects the enzymatic events in transcription we performed *in vitro* transcription assays using HeLa nuclear extracts (Dignam *et al.,* 1983) and a plasmid, pTIGL, containing a G-free transcription cassette driven by a minimal Ad major late promoter (AdMLP) (Wang *et al.,* 1998). We used the AdMLP-driven transcription template rather than an Ad *E2a* promoter because of the weakness of the *E2a* promoter in *in vitro* assays (Dignam *et al.,* 1983; Lee and Roeder, 1981; Fire *et al.,* 1981). Two versions of the AdMLP template were prepared. One version was a 3104-bp, *Xba*I-linearized fragment and the other was the same plasmid that was left uncut by restriction endonuclease. *In vitro* transcription in the presence of increasing amounts of a His-tagged Rep68 protein (Young *et al.,* 2000) resulted in elimination of the 388-nt RNA runoff transcript from the linearized plasmid (Fig. 8). However, similar amounts of Rep68 that contains a mutation in the purine nucleotide binding site (Rep68-PNB) did not repress transcription. The highest concentration of Rep68 also inhibited transcription from the circular pTIGL plasmid, whereas Rep68PNB did not affect transcription (Fig. 8, lanes 14–17). Transcription from the AdMLP template was due to RNA polymerase II because it was completely inhibited by 1 μg/ml α-amanitin (Fig. 8, lanes 11 and 12). The mechanism of this inhibition remains to be elucidated. However, it is worth noting that the pTIGL plasmid contains two regions that are homologous to the AAV Rep78/68 binding site found in the viral terminal repeat elements. The distal site has been shown to bind Rep68 in electrophoretic gel mobility shift assays (McCarty *et al.,* 1994). Therefore it is possible that Rep68 binding to the template DNA via the Rep binding sites is necessary for inhibition of transcription. That the PNB mutant had no effect on transcription suggests that ATP hydrolysis or DNA/DNA or DNA/RNA helicase activity is essential for suppression.

The experiments presented here have shown that all four Rep proteins can suppress gene expression from the Ad *E2a* transcription promoter in Ad-transformed 293 cells. These studies emulate the early stages of an Ad and AAV co-infection because of the presence of the endogenous *E1a* gene in 293 cells and the introduced *E2a* and *rep* genes. Other results from our lab have demonstrated that AAV and *rep* expression inhibits Ad *E2a* expression at the protein and mRNA levels during AAV and Ad co-infection (Jing *et al.,* 2001). These studies define which of the four Rep proteins may play roles in suppression of *E2a* expression in the presence of *trans*-activating E1a proteins. The carboxyl terminus of Rep78/52 would seem to be required for the full effect of mRNA suppression *in vivo.* In the absence of the carboxyl terminus, the PNB domain in Rep68/40 is necessary for the *in vivo* mRNA effects and for Rep68, the *in vitro* effects as well. The results in Figs. 3–6 and 8 suggest that the PNB domain's role in suppression of gene expression is primarily at the level of transcription. Luciferase and E2a protein levels increase when the

PNB mutants are included in transfection compared to the normal *rep* genes. These increases in expression probably result from the increase in mRNA that is evident in the Northern analyses shown in Fig. 5. In summary, the carboxyl terminus is required for full mRNA suppression of *E2a* expression. The PNB domain augments the mRNA effect and apparently plays only a minimal role, if any, in suppression of translation.

In an AAV and Ad co-infection, or in Ad-infected Neo6 cells, there is a two- to threefold decrease in *E2a* mRNA (Jing *et al.,* 2001). This level of inhibition is comparable to the results shown in Figs. 5 and 6 in which we observed a two- to threefold decrease in *E2a* mRNA in the presence of Rep78 and Rep52. Our results are somewhat similar to those of others in that Rep78 is a strong suppressor of heterologous gene expression and Rep40 is the weakest suppressor (Horer *et al.,* 1995). However, differences remain in the inhibitory capabilities of the Rep proteins because Rep52 is a strong suppressor of *E2a* mRNA levels but had only minimal effects on mRNA from HIV and HPV transcription promoters. The PNB site in the Rep proteins increases the inhibitory capabilities but is not the only functional domain required for inhibition of *E2a* expression.

The Rep proteins have been reported to associate with a variety of cellular proteins that are involved in the regulation of transcription. All three of the predominant transcription factors that bind to the *E2a* promoter have been reported to be affected by the Rep proteins. Rep78 and Rep52 associate with protein kinase X (PrKX) of the protein kinase A family (Chiorini *et al.,* 1998; DiPasquale *et al.,* 1998). Rep/PrKX interaction inhibits kinase action on the ATF transcription factor that in turn prevents activation of genes containing ATF regulatory elements. Rep78 has been reported to bind to TATA box binding protein (TBP) as well as disrupt TBP binding to the TATA element (Hermonat *et al.,* 1998; Su *et al.,* 2000). Recently it has been suggested that Rep expression alters Rb function and activation of *E2F* gene expression (Batchu *et al.,* 2001). Removal of each of these transcription factor binding sites did not eliminate Rep-mediated suppression of reporter gene expression. If for example, Rep proteins acted solely through the TATA box, then we would have expected that deletion of the TATA box would have eliminated Rep-mediated repression. That we did not lose Rep-mediated repression in the *E2a-Cat* assays suggests that the individual transcription factor binding sites are not the sole elements involved in Rep repression. It is possible that the Rep proteins may act through some other undefined element in the *E2a* promoter. However, we have not been able to detect Rep protein binding to 291 bp DNA fragment used in the *pE2a-Luc* vector (results not shown).

The mechanism of Rep-mediated suppression of gene transcription has not been well defined. That the elimination of the known transcription factor binding sites from the *E2a* promoter did not eliminate Rep-mediated repression of Cat activity suggests that Rep proteins may block transcription via interaction with other known targets in the initiation or elongation complex. These targets may be through other components of the transcription complex (e.g., PC4) or chromatin (e.g., HMG1). Rep68-mediated transcription inhibition of the minimal AdMLP in *in vitro* assays supports this general hypothesis. Rep protein association with a Rep binding element on the transcription template may be necessary for suppression of *in vitro* transcription (Zhan *et al.,* 1999). Such Rep-specific binding to the pTIGL templates may also be involved in Rep-mediated inhibition of *in vitro* transcription. The transcription templates used in this work contain one known and a second potential Rep binding site. Both the linear and the covalently closed circular pTIGL templates were inhibited by the wild-type Rep68 protein but not by the PNB mutant version or Rep68. Both proteins can bind to cognate Rep binding sites (Owens *et al.,* 1991). However, the actinomycin experiments shown in Fig. 6 imply that Rep78/52 inhibit *E2a* gene transcription. Rep52's inability to bind to known Rep binding sites argues against the requirement for Rep binding to the template as a means to inhibit transcription. Clearly, further experiments are required to identify the mechanism of Rep-mediated inhibition of transcription.

An additional insight into the mechanism of Rep suppression of *in vitro* transcription comes from our results showing the requirement for the purine nucleotide binding site. This requirement suggests that ATPase or helicase activities are required for transcription suppression. A functional PNB domain may be an essential requirement for transcription suppression because the Rep68PNB mutant did not decrease *E2a* mRNA levels in plasmid transfection assays (Fig. 5). The PNB mutant is still capable of binding to Rep binding sites (Owens *et al.,* 1991), therefore Rep binding alone is insufficient for transcription suppression. A possible mechanism to explain how Rep68 blocks AdMLP transcription *in vitro* is that the protein binds to one or both of the Rep binding sites and induces denaturation of the template, thus disrupting transcription. Experiments in which the Rep binding sites have been removed from the transcription template and Rep52 and Rep40 have been added to the *in vitro* transcription cocktail are currently under way.

The studies presented here provide new insights into how AAV and the Rep proteins interact with Ad. There are several potential Rep binding sites in the Ad2/5 genome. It is conceivable that Rep binding to one or more of these sites affects Ad gene expression. However, the effects of Rep52 on *E2a* gene expression argue against a simplistic explanation of Rep-mediated inhibition of Ad gene expression through site-specific DNA binding. Further experiments are required to fully define AAV's relationship with its most efficient helper virus.

MATERIALS AND METHODS

Cells, plasmids, and antibodies

Human 293 cells were originally obtained from the American Type Culture Collection and were grown in Eagle minimum essential medium supplemented with glutamine, penicillin (50 mg/ml), streptomycin (50 mg/ml), and 10% fetal bovine serum. All cells were maintained as monolayer cultures at 37°C in a 5% CO_2 atmosphere.

The pAd5E2a plasmid was constructed by removing a 5768-bp *Bam*HI to *Eco*RI DNA fragment from Ad5 (nt 21,563–27,331) and inserting it into the corresponding sites in pBluescript SK (Stratagene). Plasmid pRep1097 contains a *Xho*I linker insertion mutation at nucleotide 1097 in the AAV2 genome. It does not express any Rep protein (Yang *et al.,* 1992, 1994). Plasmid pCDMRep78G contains the wild-type Rep gene (nt 263–2233 in the AAV2 genome) cloned in pCDM8 (Invitrogen, Inc.) under the control of CMV early promoter (Yang *et al.,* 1994). The G refers to the presence of a Gly codon in place of the Rep52/40 Met initiation codon at nucleotide 992 (Chejanovsky and Carter, 1989). The pCDMRep68G plasmid was constructed by inserting the carboxyl-terminus-encoding region of Rep68 from pNTC28 into the pCDMRep78G plasmid. The pNTC28 plasmid does not encode Rep78 or Rep52 because it lacks the major p40 mRNA intron from nt 1908 to 2228 (Chejanovsky and Carter, 1989). The pCDMRep52 plasmid was constructed by removal of AAV *rep* gene sequences 263–971 from pCDMRep78. The pCDMRep40 plasmid was constructed from pCDMRep52 in a manner analogous to the construction of pCDMRep68G. The PNB site mutant versions of the Rep-expressing plasmids were constructed by replacing the normal version of the PNB with an appropriate restriction endonuclease fragment from pNTC23 which contains a Lys → His change at nt 1338–1340 in the AAV2 (Chejanovsky and Carter, 1990).

Plasmid p*E2a-Luc* contains a 291-bp *E2a* promoter element attached to a luciferase reporter gene (Jing *et al.,* 2001). Plasmid p*E2awtCAT* containing the Ad *E2a* early promoter upstream of a *Cat* reporter gene and its corresponding E2F and ATF mutants were obtained from Dr. J. Brady (Loeken and Brady, 1989). The E2F mutant lacks nucleotides −64/−60 and −40/−36 and the ATF mutant lacks nucleotides −80/−76 of the Ad E2a promoter. We constructed a TATA box deletion mutant by annealing the following oligonucleotides, which lack the TATA element: 5′-GAT CTA GCG CGC AGT ATT TGC TGA AGA GAG CCT CCG CGT CTT CCA GCG TCG GCC GAA GCT GAT CT-3′ and 5′-GAT CAG ATC AGC TTC GGC GCA CGC TGG AAG ACG CGG AGG CTC TCT TCA GCA AAT ACT GCG CGC TA. Plasmid pE2awtCAT was cut with *Bcl*I and *Bgl*II and ligated to the annealed TATA-less oligonucleotides.

Plasmid pTIGL containing the AdMLP upstream of a G-less cassette was a gift from Dr. M. Sawadogo (Wang *et al.,* 1998).

The pStump68 plasmid was kindly provided by Dr. R. J. Samulski (Young *et al.,* 2000). To construct pStump68His-PNB, a 383-bp *Bam*HI–*Sal*I DNA fragment containing *rep* gene sequences from 1045 to 1428 was removed from pCDMRepG68-PNB. The DNA fragment contains the Lys → His coding change at nt 1338–1340. The 383-bp fragment was inserted into *Bam*HI/*Sal*I-cut pStump68His. The resulting pStump68His-PNB plasmid was transformed in SG13009 cells, which carry the *Escherichia coli lacI* gene and allow for inducible expression using IPTG (Qiagen).

Antibody against Ad 72-kDa DNA binding protein, a mouse monoclonal IgG (MAb 37-3) was kindly provided by Dr. I. Kovesdi of GenVec, Inc. AAV Rep protein-specific polyclonal antibodies were obtained from rabbits immunized with a recombinant Rep protein expressed from *E. coli* (Trempe *et al.,* 1987). Alkaline phosphatase-conjugated anti-rabbit IgG (Cat. No. 31340) was obtained from Pierce Co.

RNA isolation and Northern blot analysis

Human 293 cells (5×10^6) in 100-mm dishes were cotransfected with 5 μg of pAd5E2a and 10 μg of various pCDMRep protein-expressing plasmids using the calcium phosphate method (Ausubel *et al.,* 1989). Forty-eight hours posttransfection total RNA was isolated using Trizol reagent according to the manufacturer's protocol. Thirty micrograms of total RNA was separated by formaldehyde agarose gel electrophoresis (Ausubel *et al.,* 1989). RNA was transferred to a nitrocellulose membrane and was crosslinked to the membrane using a UV Stratalinker at a setting of 1200 μJ $\times$ 100. RNA on the filter was hybridized to an α-^{32}P-labeled DNA probe that was labeled using the random-prime method according to the manufacturer's protocol (Boehringer Mannheim). Prehybridizations, hybridizations, and washings were performed as described (Ausubel *et al.,* 1989). The blot was exposed to X-ray film or to a Molecular Dynamics phosphorimager. The amount of hybridization signal was determined by the phosphorimager software or densitometric scanning of X-ray film using a Kodak Image Station 440CF and Kodak Digital Science 1D image analysis software.

Steady-state level of mRNA of Ad5*E2a* gene

293 cells were cotransfected as described above with pAd5E2a and pCDM8, pCDMRep78, or pCDMRep52. Forty hours posttransfection, actinomycin D was added to each dish at a concentration of 5 μg/ml. The cells were harvested 0, 2, 4, 8, and 12 h later and total RNA was isolated and analyzed by Northern hybridization described above.

Protein extraction and immunoblot analysis

293 cells (1×10^6) grown in six-well dishes were cotransfected using the calcium phosphate precipitation procedure with 1 μg of pAd5E2a and 2 μg of each of the

different AAV Rep plasmids. For examination of Rep protein expression, the pAdE2a plasmid was not cotransfected. Cells were harvested 48 h later and pelleted by centrifugation at 1000 *g* at 4°C for 5 min. The cell pellet was lysed on ice for 10 min in 200 μl of STM-NP buffer (25 mM sucrose, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF, and 0.1 mM DTT). The lysate was centrifuged at 2000 *g* at 4°C for 5 min to obtain a nuclear pellet. The nuclear pellet was resuspended in 200 μl of IPP buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 1 mM PMSF, 1 μM leupeptin, 1 mM benzamidine, and 1 μg/ml pepstatin A) and incubated on ice for 30 min. The nuclear extract was centrifuged at 10,000 *g* at 4°C for 10 min to remove DNA and nuclear debris. The supernatant was used as the nuclear extract. The cytoplasmic protein content was measured using a Bio-Rad protein assay kit.

Nuclear extracts containing equal amounts of protein were analyzed for E2a, Rep52, and Rep40 expression using SDS–PAGE and immunoblots as described below. To determine the level of Rep78, Rep78-PNB, Rep68, and Rep68-PNB expression, we performed immunoprecipitations followed by SDS–PAGE and immunoblot analysis. The Rep-specific antibody occasionally detects a cross-reacting cellular protein that comigrates with Rep68 (Trempe *et al.,* 1987). This contaminant can be eliminated from the gels by prior immunoprecipitation. Immunoprecipitation of Rep78/68 proteins was carried out by first preadsorbing 175 μl of affinity-purified Rep antibody to 75 μl of protein A–agarose beads overnight at 4°C with continuous rotation. The nuclear extract (equivalent to approximately 1.2×10^6 nuclei) was treated with SDS to a final concentration of 1% (w/v) and β-mercaptoethanol to a final concentration of 50 mM. The extract was heated at 70°C for 10 min, cooled, and diluted 10-fold with IPP buffer. The nuclear extract was incubated with the antibody-bound beads overnight at 4°C. The beads were pelleted by centrifugation at 500 *g* at 4°C for 5 min. The beads were washed with IPP buffer and washed three additional times. After the final wash, the beads were resuspended in SDS–PAGE sample buffer, boiled, and pelleted and the released protein was separated on a 10% SDS–PAGE gel. After electrophoresis the protein was transferred to a nitrocellulose membrane using a Bio-Rad semidry transfer unit and immunoblots were performed using Rep- or E2a-specific antiserum as described previously (Yang *et al.,* 1994).

Analysis of *E2a* reporter gene assays

293 cells (1×10^6) in six-well dishes were cotransfected using the calcium phosphate precipitation procedure with 1 μg of p*E2a-luc* or p*E2a-Cat* plasmid and 2 μg of the individual Rep-expressing plasmids. Forty-eight hours posttransfection, cells were harvested, total cellular extracts prepared, and protein concentrations determined using the Bio-Rad DC assay. Luciferase activity was determined using a luciferase reporter assay system according to the manufacturer's protocol (Promega). Cat enzyme assays were performed and analyzed by thin-layer chromatography as described (Ausubel *et al.,* 1989).

Purification of Rep68 and Rep68PNB

Purification of Rep68 from *E. coli* cultures containing pStumpRep68His was accomplished according to Young *et al.* (2000), with some modifications. One-liter cultures of pStumpRep68 or pStumpRe68-PNB-containing *E. coli* were grown and induced and cellular lysates prepared as described by Young *et al.* The crude cell lysates were collected, glycerol was added to 20% (v/v), and the lysates were applied to a Ni^{2+}-nitriloacetic acid (NTA) column (bed volume 2.5 ml) that was equilibrated with 50 mM Na_2HPO_4, pH 8.1, 1 M NaCl, 0.1% Tween 20, 10 mM β-mercaptoethanol (BME), 50 mM imidazole, pH 7.0, and 20% (v/v) glycerol. The column was washed with 5 column volumes of 50 mM NaH_2PO_4, pH 6.0, 1 M NaCl, 0.1% Tween 20, 10 mM BME, 100 mM imidazole, pH 7.0, 20% (v/v) glycerol, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, and 0.1 mM PMSF. Rep68His was eluted with a linear gradient of 0.1 to 1 M imidazole in Na_2HPO_4, pH 8.1, 20% (v/v) glycerol, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 0.1 mM PMSF, 10 mM BME, 1 M NaCl, and 0.1% Tween 20. The column fractions were collected, quickly frozen in dry ice, and stored at −70°C, after aliquots were taken for gel electrophoresis and Western blot analysis. Column fractions containing Rep68 were thawed, combined, and dialyzed against 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 20% (v/v) glycerol, 0.1 mM EDTA, 0.1% Tween 20, and 10 mM BME at 4°C for 2 h. The buffer was changed twice and final dialysis of the sample was done in the same buffer except that BME was substituted with 0.1 mM DTT. The dialyzed sample was applied to a Q-Sepharose column (bed volume 10 ml) equilibrated with 50 mM Tris–HCl, pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The column was washed with 10 bed volumes of the equilibration buffer and eluted with a linear gradient made of 50 mM–1 M NaCl in equilibration buffer at a flow rate of 1 ml/min. Column fractions were collected and their optical densities were determined at λ_{280}. Equal aliquots were taken from each fraction for gel electrophoresis and Western blot analysis and the fractions were stored at −70°C. Fractions of interest were combined and concentrated in an Amicon concentration device (mol wt cut-off 50,000) and subjected to a Sephacryl S-300 gel filtration column (bed volume 40 ml) that was equilibrated with 50 mM Tris–HCl, pH 7.5, 20% (v/v) glycerol, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions were collected and analyzed by SDS–PAGE

immunoblot analysis to identify fractions containing the Rep68His protein.

Rep68PNB was purified by following the above protocol except that the protein was passed over two Ni^{2+}-NTA columns instead of the Q-Sepharose and Sephacryl 300 columns.

In vitro transcription of Ad MLP

HeLa nuclear extracts were purchased from Promega or were prepared following published procedures (Dignam *et al.,* 1983). All steps of the *in vitro* reactions were performed as recommended in the manufacturer's protocol (Promega Corp.) using [α-^{32}P]UTP (3000 Ci/mmol). *Xba*I-linearized pTIGL (1.2 μg) or 0.2 μg of circular pTIGL was used as transcription template. Purified Rep68 or Rep68-PNB was added to final concentrations of 0.032, 0.064, 0.096, and 0.128 μg/μl. *In vitro*-synthesized RNA was phenol–chloroform and chloroform extracted, ethanol precipitated, and analyzed by gel electrophoresis in a 5% polyacrylamide gel containing 7 M urea, 45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.0. The gel was then dried and exposed to X-ray film.

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