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Repression of RNA Polymerase III Transcription by Adenovirus E1A

KERSTIN SOLLERBRANT, GÖRAN AKUSJÄRVI, AND CATHARINA SVENSSON*

Division of Microbial Genetics, Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden

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Adenovirus E1A encodes two major proteins of 289 and 243 amino acids (289R and 243R), which both have transcription regulatory properties. E1A-289R is a transactivator whereas E1A-243R primarily functions as a repressor of transcription. Here we show that E1A repression is not restricted to RNA polymerase II genes but also includes the adenovirus virus-associated (VA) RNA genes. These genes are transcribed by RNA polymerase III and have previously been suggested to be the target of an E1A-289R-mediated transactivation. Surprisingly, we found that during transient transfection both E1A proteins repressed VA RNA transcription. E1A repression of VA RNA transcription required both conserved regions 1 and 2 and therefore differed from the E1A-mediated inhibition of simian virus 40 enhancer activity which primarily required conserved region 1. The repression was counteracted by the E1B-19K protein, which also, in the absence of E1A, enhanced the accumulation of VA RNA. Importantly, we show that efficient VA RNA transcription requires expression of both E1A and the E1B-19K protein during virus infection.

Two proteins of 243 and 289 amino acids (243R and 289R) encoded by the adenovirus E1A region have the potential to reprogram cellular growth because of their ability to positively and negatively modulate gene expression. A large number of studies on this topic have led to the current view that the E1A-289R protein mainly functions as a transcription activator whereas the E1A-243R protein primarily acts as a transcription repressor (reviewed in reference 7). The two proteins are identical with the exception of 46 internal amino acids present only in the E1A-289R protein (42). These amino acids correspond to the conserved region 3 (CR3) and constitute the minimal transactivating domain of E1A (18, 35). Two additional conserved regions, designated CR1 and CR2, are required for the transcription repression and the transforming capacity of E1A (33, 47).

Although E1A-289R is regarded as the main transactivator, E1A-243R is not completely devoid of transactivating potential. For example, activation of the E2F transcription factor through dissociation of transcription inhibitors requires CR1 and CR2 (1, 9, 44), and E1A-243R has also been shown to activate transcription of certain cellular genes (30, 37, 56).

Besides their transactivating capacity, the E1A proteins can repress transcription of several viral and cellular RNA polymerase (Pol) II genes (reference 46 and references therein; 8, 28, 29, 40, 68). The mechanism for repression is not known, but it has been shown in one case that the E1A proteins can block formation of transcription complexes (28), possibly by interfering with the interaction between basal and upstream binding transcription factors. The regions required for transrepression have been mapped mainly to CR1 and the amino terminus of the E1A protein (26, 47). Since these regions are also required for the binding of a cellular DNA-binding 300K protein, a functional correlation between transcription repression and 300K binding has been suggested (38, 45, 51, 53). However, transrepression by E1A is probably more complex, involving several independent mechanisms, since CR2 and sequences in the carboxy-terminal exon also are important for transrepression of certain genes and transcription factors (35, 36, 58, 68).

E1A has also been implicated in the transcription control of RNA Pol III genes such as a *Drosophila* tRNA arginine gene and the adenovirus virus-associated (VA) RNA I and II genes (5, 16, 23, 67). Transcription of the VA RNA genes is under the control of two intragenic promoter elements (15) and requires at least two transcription factors (TFIIIB and TFIIIC) (31). TFIIIC has been suggested to be the target for an E1A-mediated transactivation (22, 67). By in vitro transcription in cell extracts prepared from adenovirus-infected cells, transactivation of VA RNA transcription was shown to correlate with an increase in the amount of the active form of TFIIIC (22, 67). TFIIIC exists in two interconvertible forms differing in their phosphorylated state, and an adenovirus infection can induce an E1A-dependent increase in the hyperphosphorylated form of TFIIIC (23). The shift from an inactive to an active form of TFIIIC-DNA complex has also been reproduced in uninfected nuclear extracts, supplemented with exogenously produced E1A-289R protein (11, 41).

A majority of reports have identified viral and cellular enhancers as targets for E1A-243R transrepression, but also more simple RNA Pol II promoters are repressed (12). Furthermore, the TATA-binding protein (TBP) of the classical RNA Pol II transcription factor TFIID has recently been demonstrated to be involved in RNA Pol I and III transcription (10, 48, 61). Since transrepression probably is a consequence of E1A interaction and/or modification of transcription factors, we decided to investigate whether E1A could also repress RNA Pol III transcription. Surprisingly, both the E1A-243R and E1A-289R proteins were capable of repressing adenovirus VA RNA I and II transcription during transient transfection. Efficient E1A transrepression required both CR1 and CR2. Furthermore, the E1B-19K product was demonstrated to enhance basal VA RNA expression as well as to counteract E1A-mediated transrepression of VA RNA transcription. The transactivation potential of the E1B-19K protein was also reproduced in virus infec-

* Corresponding author.

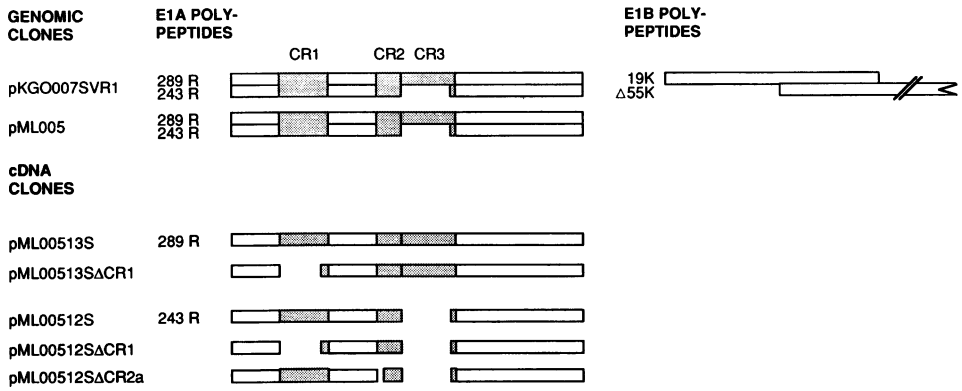


FIG. 1. Protein-coding capacity of the E1A plasmids. Locations of the E1A conserved regions (CR1 to -3) are indicated. The two genomic clones pKGO007SVRI and pML005 encode both major E1A polypeptides (E1A-243R and E1A-289R). pKGO007SVRI in addition encodes the E1B-19K polypeptide and a truncated form of E1B-55K (Δ55K). cDNA clones encode the E1A-243R and E1A-289R proteins individually, and mutants thereof encode proteins lacking the major part of CR1 (ΔCR1, amino acids 38 to 65) or the N-terminal region of CR2 (ΔCR2a, amino acids 121 to 125).

tions since an adenovirus E1B-19K mutant was severely defective in the accumulation of VA RNA.

MATERIALS AND METHODS

Plasmid DNA. pKGO007SVRI has been described elsewhere (54). pML005 was created by replacing the *Sac*I site (nucleotide [nt] 1766)-to-*Hin*dIII (nt 2798) fragment in pKGO007SVRI with a *Hin*dIII linker. pML00512S and pML00513S were constructed by replacing the *Sac*II (nt 356)-*Xba*I (nt 1336) and *Bsp*MII (nt 826)-*Xba*I (nt 1336) fragments, respectively, of pML005 with the corresponding fragment spanning the splice junction site from cDNA clones pKGO12S and pKGO13S (54), respectively. The ΔCR1 and ΔCR2a mutations were originally described as deletion mutants G5/3 and GCX, respectively (47). These mutations were initially transferred into the pKGO007SVRI background (53) and then further transferred into cDNA clones pML00512S and pML00513S, creating pML00512SΔCR1, pML00512SΔCR2a, and pML00513SΔCR1. pSXβ+ expresses the rabbit β-globin gene under the transcription control of the simian virus 40 (SV40) enhancer (2). The three VA RNA-expressing constructs, pVA I, pVA II, and pHindB, have all been described previously (52). The three E1B gene-expressing constructs express the individual E1B-19K and E1B-55K products, pCMV19K and pCMV55K, respectively, or the complete E1B transcription unit, pCMVE1B, under the transcription control of the cytomegalovirus promoter-enhancer (59). pE4CAT (34) was kindly provided by M. Green. pE4globin was constructed by inserting an *Xba*I-*Hae*III fragment from pE4CAT, spanning the adenovirus type 5 E4 promoter, upstream of a promoterless rabbit β-globin gene. pVA II/I encodes a hybrid VA RNA product and was created by fusing the 5′ portion of VA RNA II (nt 10865 to 10930) to the 3′ portion of VA RNA I (nt 10680 to 10764).

Cell culture conditions and transfection. HeLa and Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. Transfections were done on 60-mm petri dishes by the calcium phosphate coprecipitation technique essentially as described in reference 64. The amounts of transfected reporter and effector plasmids are indicated in the figure legends. Whenever necessary, the total amount of transfected plasmid DNA was adjusted to 10 to 13 μg with pUC19 carrier DNA. Approximately 48 h after transfection, cells were harvested, the plasma membrane was lysed by treatment with IsoB–0.65% Nonidet P-40, and the cytoplasmic RNA fraction was purified by extraction with phenol-chloroform.

S1 analysis. Conditions for the S1 endonuclease analysis have been described elsewhere (54). The 5′-end-labeled VA RNA I, VA RNA II, and β-globin DNA probes were as described previously (2, 52). The VA RNA II/I-specific probe was end labeled at the *Bst*EII site in pVA II/I, recleaved, and isolated as a 939-bp fragment. Following S1 analysis, a 94-bp fragment is protected. A total of 5 or 10 μg of cytoplasmic RNA was analyzed. The hybridization temperature was 50°C. After the S1 endonuclease digestion, the protected fragments were separated through a 6% denaturing polyacrylamide gel, and the amount of radioactivity was quantitated with the ImageQuant computer program on a Phosphorimager (Molecular Dynamics).

CAT assay. Chloramphenicol acetyltransferase assays were performed essentially as described in reference 17. The amount of radioactivity was quantitated with the ImageQuant computer program on a Phosphorimager (Molecular Dynamics).

Viruses and virus infection. Viruses were grown and titrated on HeLa or 293 monolayer cells. Viruses *wt*900 and *dl*902 (55), *dl*312 (27), and *pm*1722 (3) have been described previously. For combined infection-transfection experiments, cells were infected at a multiplicity of 10 fluorescence-forming units per cell (43). To prevent viral DNA replication, AraC was added at 1 h postinfection to a final concentration of 25 μg/ml and replenished every 10 h throughout the infection. At 13 h postinfection, cells were transfected with the reporter plasmids, and at 40 h postinfection, cytoplasmic RNA was prepared as described above.

RESULTS

E1A inhibits VA RNA transcription. To study the effects of the E1A-289R and E1A-243R proteins on VA RNA transcription, we used plasmid cotransfection into HeLa cells. Cotransfection with plasmid pKGO007SVRI (Fig. 1), expressing E1A as well as the 5′ half of E1B, with reporter

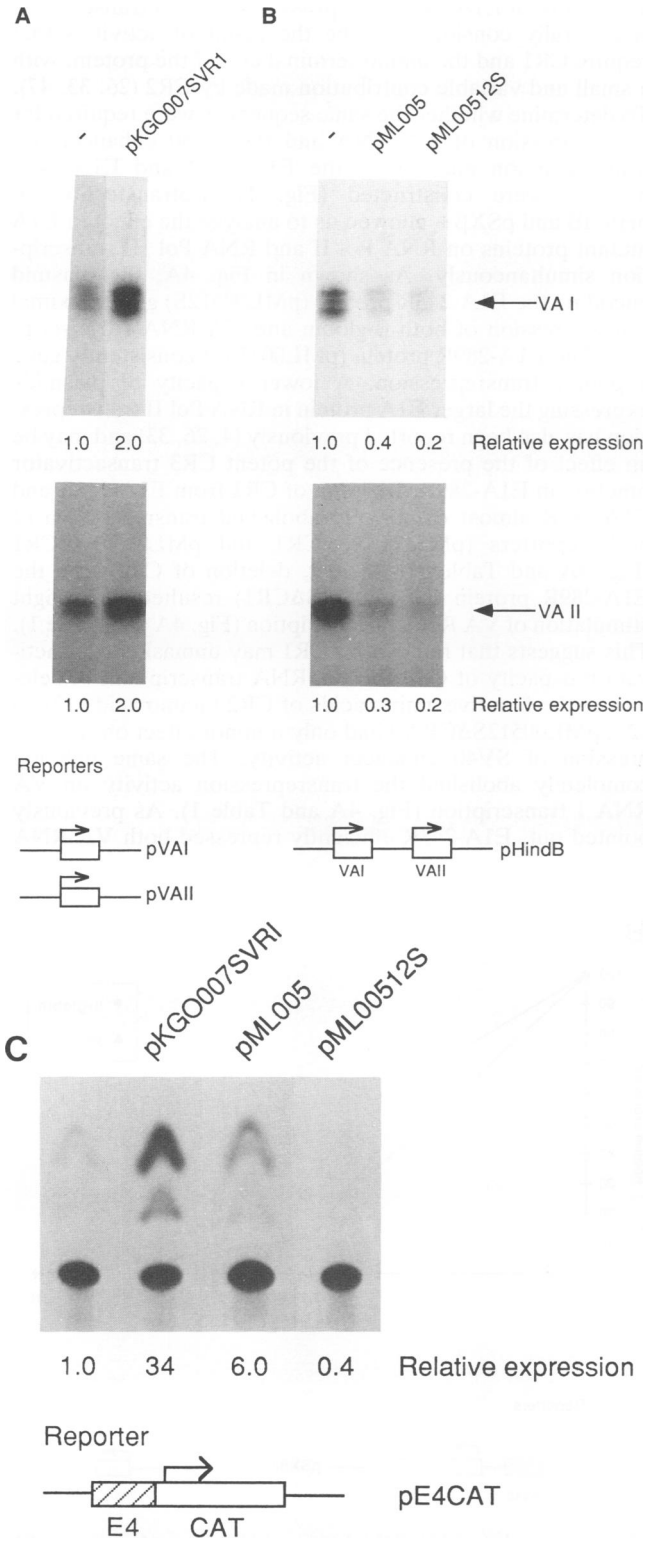


FIG. 2. Activation (A) and repression (B) of VA RNA transcription mediated by the adenovirus E1 gene products. HeLa cells were transfected with 1 μg of the VA RNA-encoding plasmids delineated at the bottom of the figure, alone (lanes −) or together with 5 μg of E1-encoding plasmid. At 48 h posttransfection, cytoplasmic RNA was subjected to S1 analysis with end-labeled VA RNA I and II probes. Protected fragments, indicated by arrows, were separated on a 6% denaturing polyacrylamide gel. (C) Effect of the E1 products on expression from the E4-CAT construct. HeLa cells were transfected with 2.5 μg of the reporter plasmid pE4CAT either alone or together with 2.5 μg of an E1-expressing plasmid as indicated. At 48 h posttransfection, cytoplasmic extract was harvested and analyzed for CAT activity.

plasmids encoding the adenovirus VA RNA genes, resulted in an approximately twofold increase in both VA RNA I and VA RNA II transcription (Fig. 2A and Table 1). This was consistent with previous results (23), although the level of stimulation in our study was much lower. The E1A-243R protein is believed to be the major E1A protein with RNA Pol II transcription repression activity (reference 4 and references therein). Since the E1B region has been reported to counteract E1A-dependent transrepression of RNA Pol II genes (39, 66), two plasmids which were devoid of E1B sequences were constructed: pML00512S, expressing the E1A-243R protein, and pML005, encoding both major E1A proteins (Fig. 1). Cotransfection of pML00512S and pHindB, expressing both adenovirus VA RNA genes, resulted in a significant reduction of VA RNA I and VA RNA II transcription (Fig. 2B and Table 1). Surprisingly, pML005, expressing both E1A-289R and E1A-243R, also failed to activate VA RNA transcription and instead repressed transcription of both VA RNA genes (Fig. 2B and Table 1). Further experiments showed that even separate expression of the E1A-289R protein (pML00513S [Fig. 1]) repressed VA RNA I expression, albeit with reduced efficiency compared with the E1A-243R protein (see Fig. 4A and Table 1). The observed lack of E1A-mediated transactivation was not caused by saturating levels of VA RNA transcription since transrepression was observed also when the amount of transfected VA RNA reporter was decreased 20-fold (0.05 μg) (data not shown). The fact that VA RNA activation could be seen only with a construct coexpressing E1A and E1B proteins (pKGO007SVRI [Fig. 1 and 2A]) prompted us to examine the effect of the two major E1B-encoded proteins on RNA Pol III transcription further (see below).

To exclude the possibility that our HeLa cell line was unresponsive to E1A transactivation, we analyzed the effect of E1A on a well-characterized target for E1A-mediated activation of RNA Pol II genes, the adenovirus E4 promoter. The ability of E1A to increase expression from an E4CAT reporter is shown in Fig. 2C. Cotransfection of a plasmid expressing the wild-type (wt) E1A gene (pML005) resulted in a sixfold increase in CAT expression, whereas expression of the E1A-243R protein alone (pML00512S) resulted in an approximately twofold repression. A significantly better transactivation was seen with pKGO007SVRI (34-fold) compared with pML005, which we interpret to be caused by the expression of the E1B-19K protein (see below).

It has been suggested that the level of induction of VA RNA expression during a virus infection is dependent on the growth conditions of the cells such that growth-retarded cells will exhibit a more pronounced E1A-dependent VA RNA activation (22). However, although prolonged serum starvation of our HeLa cells efficiently decreased basal VA RNA expression (eightfold), the relative repression caused by E1A remained similar (Fig. 3A). Furthermore, the inhibition of VA RNA transcription was also reproduced in transfected Vero cells (Fig. 4B), in T24-H-*ras*-transformed rat embryo fibroblasts, and in a separate clone of HeLa cells (data not shown). Collectively, these results strongly suggest

TABLE 1. E1-mediated modulation of RNA Pol II and III transcription[a]

E1A plasmid	VA RNA I	β-Globin
None	100	100
pML005	66	17
pML00512S	33	11
pML00512SΔCR1	96	76
pML00512SΔCR2a	86	19
pML00513S	61	19
pML00513SΔCR1	182	86
pKGO007SVRI	186	29

[a] Values represent the average RNA expression from the two reporters pHindB and pSXβ+ relative to basal expression in the absence of E1A. Data are given as mean values from at least three independent experiments.

that E1A primarily functions as a repressor of VA RNA transcription in transient transfection experiments.

E1A repression of VA RNA transcription requires conserved regions 1 and 2. The SV40 enhancer is the classical target for E1A-mediated transrepression (6, 57), and the E1A sequence requirement for transrepression has been extensively studied (4, 26, 35). To compare transrepression of RNA Pol II and III transcription, we first performed a titration experiment in which the reporter pVA I was cotransfected with increasing amounts of pML00512S in the presence of a reference plasmid, expressing the rabbit β-globin gene under the transcription control of the SV40 enhancer (pSXβ+ [2]). Maximal transrepression of VA RNA was observed already at low levels of pML00512S, whereas expression from pSXβ+ continued to decrease and eventually disappeared with increasing amounts of E1A (Fig. 3B).

The ability of E1A-243R to repress RNA Pol II transcription is generally considered to be the result of activities that require CR1 and the amino-terminal end of the protein, with a small and variable contribution made by CR2 (26, 33, 47). To determine whether the same sequences were required for transrepression of VA RNA and the SV40 enhancer, in-frame deletion mutants of the E1A-243R and E1A-289R proteins were constructed (Fig. 1). Cotransfection of pHindB and pSXβ+ allowed us to analyze the effect of E1A mutant proteins on RNA Pol II and RNA Pol III transcription simultaneously. As shown in Fig. 4A, the plasmid encoding the E1A-243R protein (pML00512S) gave maximal transrepression of both β-globin and VA RNA I transcription. The E1A-289R protein (pML00513S) consistently gave a poorer transrepression. A lower capacity of plasmids expressing the larger E1A protein in RNA Pol II transrepression has also been reported previously (4, 26, 33) and may be an effect of the presence of the potent CR3 transactivator function in E1A-289R. Deletion of CR1 from E1A-243R and E1A-289R almost completely abolished transrepression of both reporters (pML00512SΔCR1 and pML00513SΔCR1 [Fig. 4A and Table 1]). In fact, deletion of CR1 from the E1A-289R protein (pML00513SΔCR1) resulted in a slight stimulation of VA RNA I transcription (Fig. 4A and Table 1). This suggests that removal of CR1 may unmask a transactivation capacity of CR3 for VA RNA transcription. A deletion of the first five amino acids of CR2 (amino acids 121 to 125, pML00512SΔCR2a) had only a minor effect on transrepression of SV40 enhancer activity. The same deletion completely abolished the transrepression activity on VA RNA I transcription (Fig. 4A and Table 1). As previously pointed out, E1A-243R efficiently repressed both VA RNA

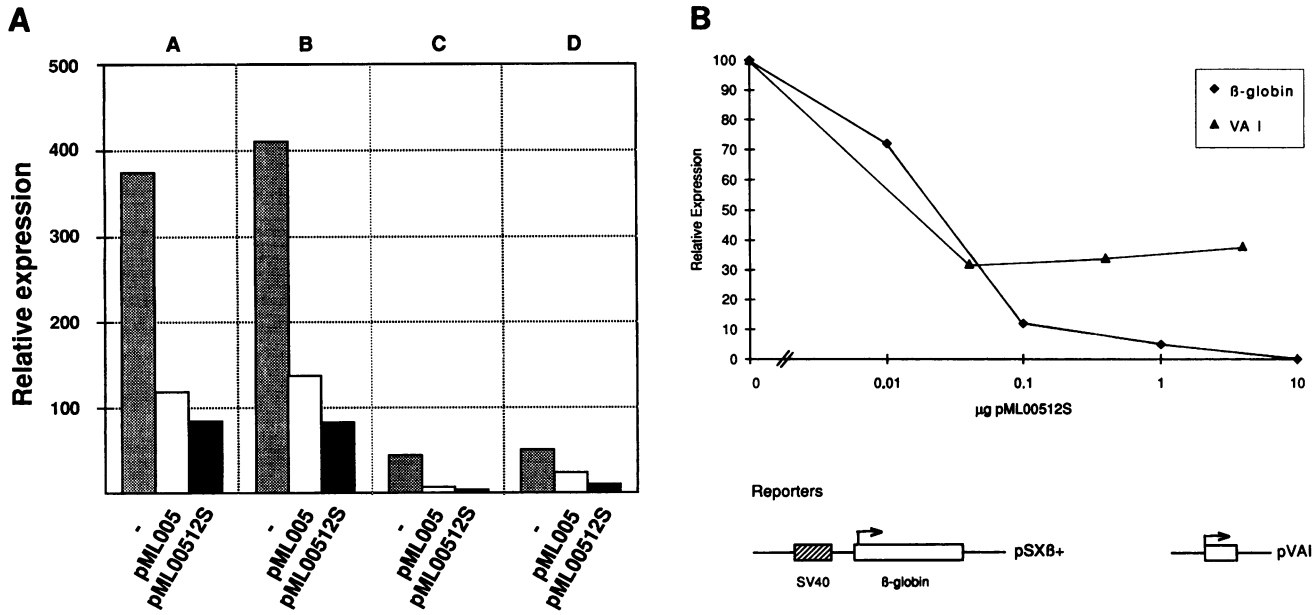
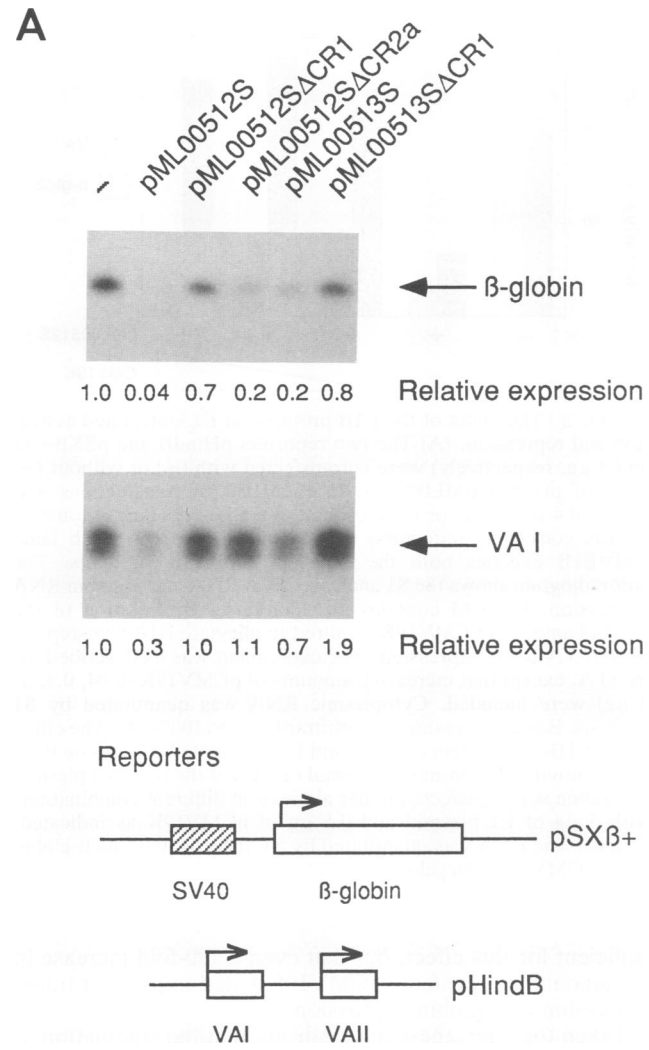


FIG. 3. (A) The effect of decreasing serum concentrations on E1A-mediated repression of VA RNA I transcription. Transfection and S1 analysis were as described in the legend to Fig. 2A and B. pHindB was used as the reporter plasmid. Prior to transfections, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (A and B) or 0.5% fetal calf serum (C and D). During the transfection, cells were supplemented with 0.5% fetal calf serum (A and C) or grown without serum (B and D). (B) Identification of the amount of E1A-243R expression required for maximal repression of the VA RNA I and SV40-driven β-globin transcription. Constant amounts of the two reporters pVAI (0.05 μg) and pSXβ+ (3 μg) were cotransfected separately with an increasing amount of plasmid pML00512S encoding the E1A-243R protein. S1 analyses were performed as described in the legend to Fig. 2. The diagram shows the expression from the two reporters relative to basal expression in the absence of E1A.



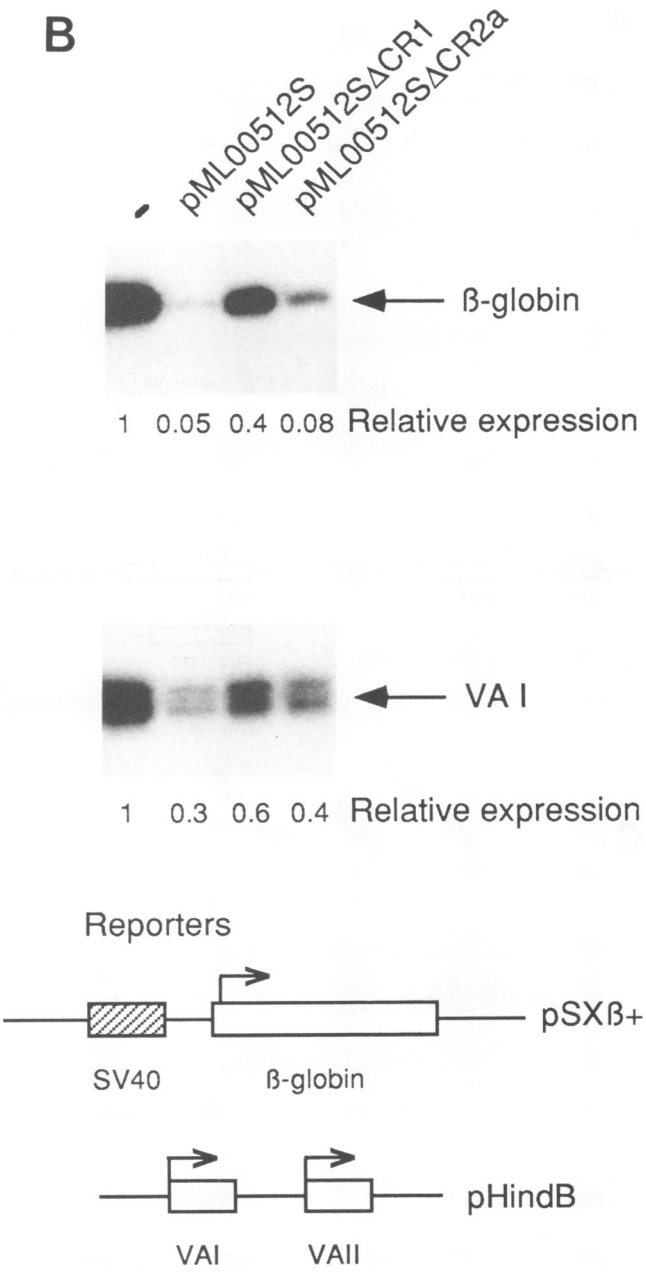
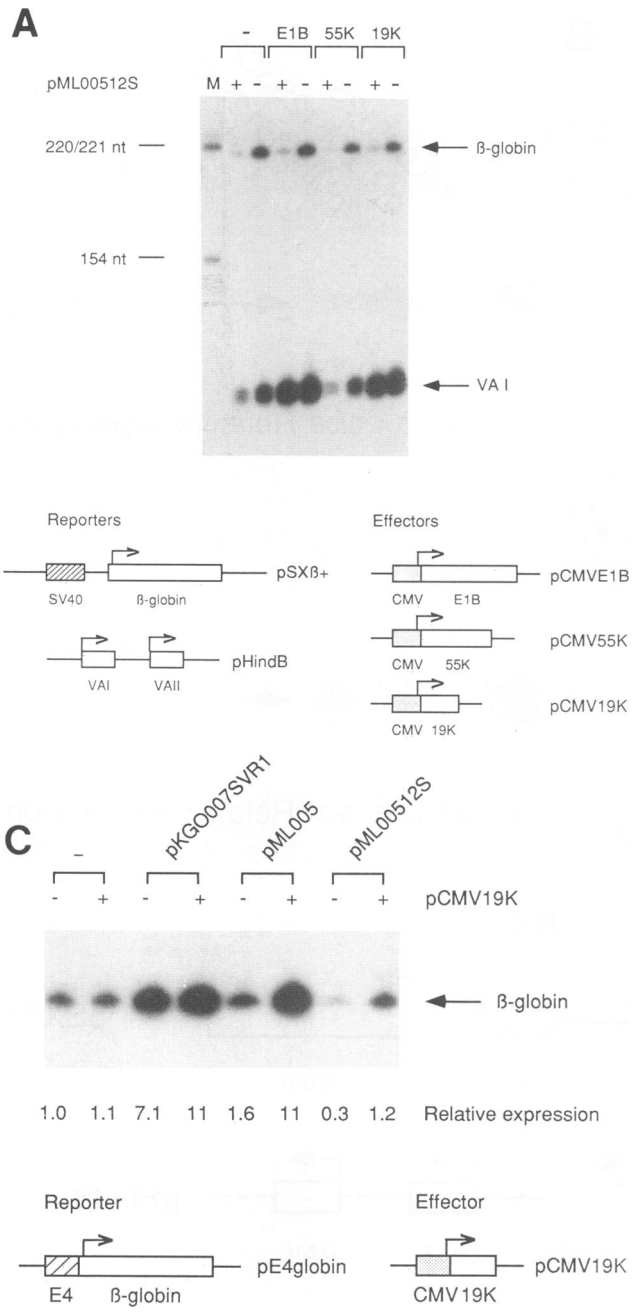


FIG. 4. Identification of the E1A domain required for transrepression of the VA RNA I gene and the SV40 enhancer-driven β-globin gene. Transfection was as described in the legend to Fig. 2A and B with the inclusion of 3 μg of the reporter pSXβ+ expressing the SV40 enhancer-driven rabbit β-globin gene (2). Cytoplasmic RNA was quantitated by S1 analysis with end-labeled DNA probes recognizing VA RNA I and β-globin RNA. (A) HeLa cells. (B) Vero cells.

and β-globin expression in Vero cells (Fig. 4B). Deletion of either CR1 or CR2 alone could not completely eliminate E1A transrepression, suggesting that additional regions in the E1A protein may affect the transrepression activity in these cells. The E1A mutants used in this study express similar amounts of protein (53). Therefore, the different transrepression capacities cannot be explained by differences in protein levels.

In summary, our results suggest that RNA Pol II and RNA Pol III transrepression are similar in that they both require CR1. However, a difference concerning the relative importance of CR2 for transrepression was seen. Whereas VA RNA I repression in HeLa cells also required CR2, SV40 enhancer repression was only slightly reduced by a CR2 deletion.

The E1B-19K protein stimulates VA RNA I expression. In contrast to previous reports, our results suggest that the E1A-289R and E1A-243R proteins primarily act as inhibitors of VA RNA transcription during transient transfection. The activation potential of plasmid pKGO007SVRI (Fig. 2A) correlated with the presence of E1B sequences. This plasmid encodes, besides E1A proteins, the E1B-19K product as well as the amino-terminal half of the E1B-55K protein. This observation implicated a function of the E1B proteins in activation of VA RNA I transcription. To investigate this possibility, three E1B-encoding expression vectors were analyzed for their ability to regulate VA RNA I transcription; pCMVE1B expressing the complete E1B transcription unit, pCMV55K encoding the E1B-55K gene, and pCMV19K encoding the E1B-19K gene (59). As shown in Fig. 5A, none of the E1B constructs affected the basal level



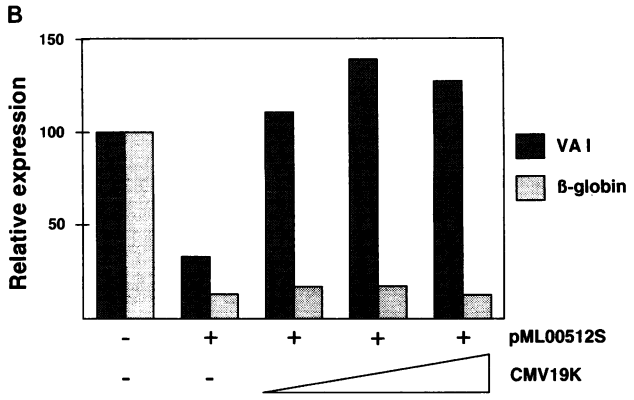


FIG. 5. The effect of the E1B proteins on E1A-mediated activation and repression. (A) The two reporters pHindB and pSXβ+ (1 and 3 μg, respectively) were cotransfected with (+) or without (−) 5 μg of plasmid pML00512S. In each pair of transfections was included 4 μg of one or none of the three E1B-encoding cytomegalovirus constructs (effectors) (59) as indicated above each lane. CMVE1B encodes both the E1B-19K and 55K products. The autoradiogram shows the S1 analysis of VA RNA and β-globin RNA expression. Lane M contains size markers. (B) Titration of the optimal amount of CMV19K required to alleviate E1A transrepression of VA RNA expression. The experiment was as described for panel A, except that increasing amounts of pCMV19K (0.04, 0.4, or 4 μg) were included. Cytoplasmic RNA was quantitated by S1 analysis. Basal expression was arbitrarily set to 100%. (C) The effect of the E1B-19K protein on basal and E1A-mediated expression from the adenovirus E4 promoter. A total of 3 μg of the reporter plasmid pE4globin was transfected either alone or in different combinations with 5 μg of E1 plasmid and 0.5 μg of pCMV19K as indicated. Cytoplasmic RNA was quantitated by S1 analysis with the β-globin probe. CMV, cytomegalovirus.

of β-globin RNA expression. In contrast, cotransfection of pCMVE1B or pCMV19K resulted in an approximately 2.5-fold increase in VA RNA I expression (Fig. 5A). This stimulation was specific for the E1B-19K product since cotransfection with pCMV55K had no effect on VA RNA I accumulation.

The effect of the individual E1B proteins on E1A-mediated repression of VA RNA I and β-globin transcription was also investigated. E1A-repressed levels of β-globin transcription were inert to both E1B proteins (Fig. 5A). In contrast, the E1B-19K, but not the E1B-55K, protein alleviated the effect of the repressor activity of the E1A-243R protein on VA RNA I transcription (pCMV19K and pCMVE1B [Fig. 5A]). Figure 5B shows that low levels of CMV19K (0.04 μg) were sufficient for this effect, but that even a 100-fold increase in the amount of transfected CMV19K failed to prevent transrepression of β-globin expression.

Taken together, these data indicate that the stimulation of VA RNA I accumulation observed after cotransfection with pKGO007SVRI is due to expression of the E1B-19K protein, with no contribution or a minor one by E1A.

As shown earlier, a stronger enhancement of E4CAT expression was seen after cotransfection with pKGO007SV RI compared with pML005 (Fig. 2C). A similar increase in E4 promoter activity was also seen when pCMV19K was cotransfected with an E4 β-globin reporter and pML005 or pML00512S (Fig. 5C), although in this particular experiment the level of E1A stimulation was lower than average (compare Fig. 2C and 5C). This indicates an involvement of E1B-19K also in RNA Pol II transcription. In contrast, only a minor effect was observed when pCMV19K was included together with pKGO007SVRI, which already encodes the E1B-19K protein. It is worth noting that whereas basal VA RNA expression was enhanced by CMV19K cotransfection (Fig. 5A), the activity of the E4 promoter was in general unaffected by the E1B-19K protein alone (Fig. 5C).

Enhancement of VA RNA expression during adenovirus infection requires E1A and E1B. Our results, suggesting that E1A inhibits VA RNA expression in transient transfection experiments, stand in striking contrast to previous reports showing that E1A activates VA RNA transcription (5, 16, 23, 67). However, the majority of earlier work has assayed the effect of E1A on RNA Pol III transcription in wt-infected cells compared with adenovirus E1A-deletion mutant-in-

fected cells, or in transcription-competent extracts prepared from wt- or mutant-infected cells (5, 16, 23, 67). Since we found that the E1B-19K protein activated VA RNA expression in transient expression assays, we decided to investigate whether VA RNA expression required E1B-19K also during a virus infection. Adenovirus-infected HeLa cells, maintained in an extended early stage of infection with AraC, were transfected with the reporter plasmid pVAII/I, expressing a hybrid VA RNA gene which easily can be distinguished from the wt VA RNAs expressed from the virus chromosome. VA RNA expression from the transfected pVAII/I was increased 52-fold during a wt adenovirus infection (Fig. 6B) compared with mock-infected cells. Infection with an E1A mutant capable of expressing only E1A-243R (*dl*902) resulted in a weaker increase (12-fold) whereas the E1A-negative mutant (*dl*312) gave only a 4-fold activation of VA RNA II/I. Approximately the same effects were seen in the accumulation of VA RNA I encoded from the virus chromosome (data not shown). To compare the effects of virus-encoded proteins on RNA Pol II and RNA Pol III transcription, the E4-globin reporter was also included in the transfection cocktail. Transcription from the E4 promoter was induced by the E1A protein expressed during an adenovirus infection in a manner very similar to that of the activation of VA RNA II/I (Fig. 6A).

In contrast, a virus mutant defective for E1B-19K production (*pm*1722 [3]) failed to efficiently accumulate both VA RNA II/I (Fig. 6B) and β-globin mRNA expressed from the transfected pE4globin reporter (Fig. 6A). Expression of VA RNA II/I was significantly more sensitive to the absence of E1B-19K than was E4-globin expression. In fact, expression of VA RNA II/I in *pm*1722-infected cells was even lower than during a *dl*312 infection. Collectively, these results suggest that both E1A and E1B-19K expression are required for efficient VA RNA expression during a virus infection.

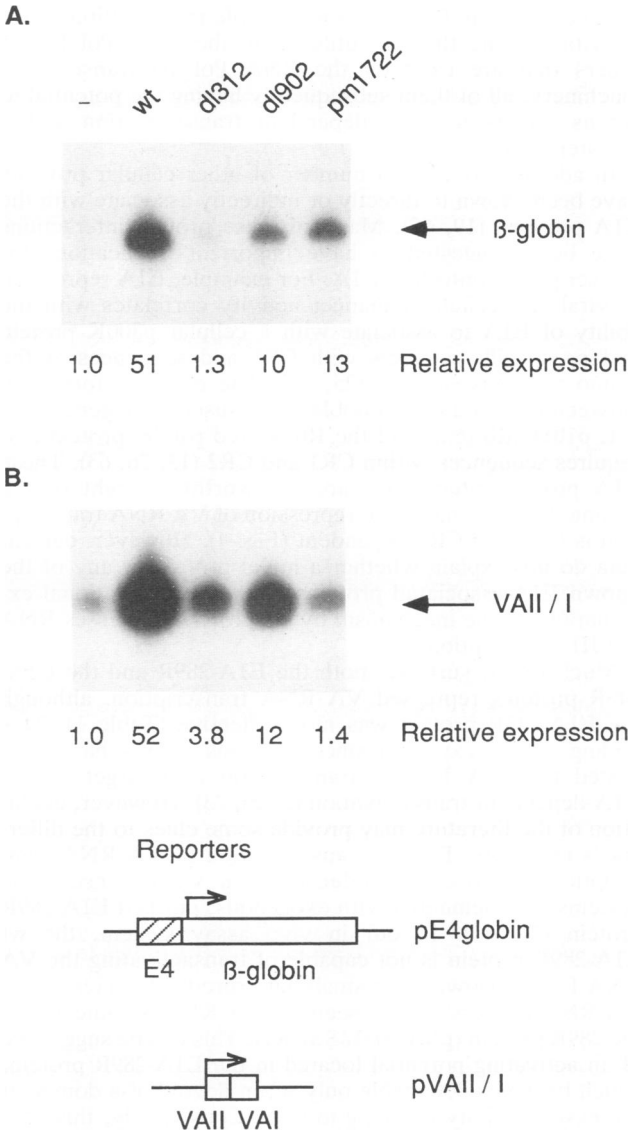


FIG. 6. The effect on RNA Pol II and III transcription during viral infections. HeLa cells were infected with 10 fluorescence-forming units per cell of *wt*900, the E1A deletion mutant *dl*312, *dl*902 expressing only E1A-243R, or the E1B-19K mutant virus *pm*1722, as indicated. DNA replication was prevented by the addition of AraC to a final concentration of 25 μg/ml. At 13 h postinfection, the two reporter plasmids pE4globin and pVAII/I (2.5 and 1.5 μg, respectively) were introduced with the calcium phosphate cotransfection technique. At 40 h postinfection, cytoplasmic RNA was prepared and quantitated by S1 analysis with end-labeled DNA probes recognizing β-globin RNA (A) or VA II/I fusion RNA (B).

DISCUSSION

Transrepression of RNA Pol II transcription by the adenovirus E1A proteins has been well documented although the mechanism(s) is still unresolved. In this report, we show that transrepression goes beyond RNA Pol II transcription and also includes transcription of the adenovirus VA RNA genes, which are transcribed by RNA Pol III. The E1A proteins have been shown to be toxic to cells and induce DNA degradation in transient transfection assays (60). Although this phenomenon might provide a plausible explanation for the repressive effect of E1A on VA RNA expression, we find it highly unlikely since we have not detected any E1A-specific DNA degradation under our experimental conditions (not shown). Furthermore, the cytotoxic effect of E1A has been mapped to CR1 (60) and we find that VA RNA repression depends on the presence of both CR1 and CR2 (Fig. 4A). The latter observation may suggest that E1A-mediated repression of VA RNA transcription and SV40 enhancer-dependent transcription differ mechanistically since inhibition of SV40 enhancer activity primarily requires an intact CR1 region (Fig. 4). This possibility is further supported by the different magnitude of transrepression of VA RNA and SV40 enhancer-driven transcription in response to increasing levels of E1A (Fig. 3B).

E1A appears to repress RNA Pol II transcription by preventing formation of stable transcription initiation complexes (28), possibly through a sequestering of transcription factors or adaptors. If E1A-mediated inhibition of VA RNA expression were to involve a similar mechanism, this could imply that a common subset of cellular transcription factors may be targets for both RNA Pol II and Pol III transrepression. In this respect, it is interesting to note that the E1A proteins can bind the TATA box binding component (TBP) of TFIID (24, 32). These contacts may be essential for both the positive and the negative effects of E1A on transcription. Although TBP was originally believed to be a unique component of RNA Pol II transcription, it has recently been shown to be required for transcription of several RNA Pol III genes, including VA RNA I (50, 61). Thus, it is tempting

to speculate that TBP exists in multiple transcription factor activities, some that are utilized by the RNA Pol II and others that are used by the RNA Pol III transcription machinery, all of them subsequently having the potential to act as targets for E1A-dependent transactivation and/or transrepression.

In addition to TBP, a number of other cellular proteins have been shown to directly or indirectly associate with the E1A proteins (19, 65). Many of these protein interactions have been suggested to have important implications for transcription control by E1A. For example, E1A repression of viral and cellular enhancer activity correlates with the ability of E1A to associate with a cellular p300K protein making specific contacts with CR1 and sequences at the amino terminus of E1A (25, 51). The complex formation between E1A and the retinoblastoma-susceptible gene product, p105K-Rb (62), and the Rb-related p107K protein (14) requires sequences within CR1 and CR2 (13, 26, 63). These E1A protein interactions are noteworthy in light of the finding that E1A-mediated repression of VA RNA transcription is CR1 and CR2 dependent (Fig. 4). However, current data do not explain whether a novel protein or any of the known E1A-associated proteins can provide a rational explanation for the mechanism by which E1A represses RNA Pol III transcription.

Much to our surprise, both the E1A-289R and the E1A-243R proteins repressed VA RNA transcription, although the E1A-243R protein was more effective (Table 1). This finding was unexpected since previous results have suggested that VA RNA I transcription is a target for an E1A-dependent transactivation (5, 16, 23). However, evaluation of the literature may provide some clues to the differences in results. Firstly, transactivation of VA RNA transcription has been reproduced in in vitro transcription systems, supplemented with exogenous, purified E1A-289R protein (11, 41). In our in vivo assay system, the wt E1A-289R protein is not capable of transactivating the VA RNA I gene. However, a small but reproducible increase in VA RNA expression was seen when CR1 was truncated in the 289R protein (pML00513SΔCR1). This can be suggestive of an activating potential located in the E1A-289R protein, which becomes detectable only when devoid of a dominant repressive activity mapping to CR1. Consistently, this activation is CR3 dependent (compare pML00513SΔCR1 and pML00512SΔCR1, Table 1). The absence of E1A-mediated repression in vitro could be explained by the loss or inactivation of essential components in transcription extracts.

Secondly, E1A has been suggested to activate VA RNA transcription by increasing the fraction of transcriptionally active, phosphorylated forms of TFIIIC (22). According to this model, our inability to detect more than a modest E1A stimulation would therefore imply that the majority of TFIIIC is already in its transcriptionally active form. However, increasing the stringency of serum starvation of growing cells, giving an eightfold reduction in basal VA RNA expression, still failed to result in an E1A-dependent activation of VA RNA transcription (Fig. 3A). Taken together, our data indicate that E1A by itself is a very poor activator of VA RNA transcription and predominantly functions as a transcriptional repressor of VA RNA during transient transfection experiments.

Finally, it is also worth pointing out that most studies of E1A-dependent activation of RNA Pol III transcription have been done by comparing the transcription activities of target genes in wt- and E1A deletion mutant-infected cells (5, 16, 22, 23, 67). Therefore, the possibility that viral functions, in addition to E1A, may contribute to the observed activation of VA RNA transcription exists. In agreement with this, we find that the E1B-19K protein activates VA RNA expression in a transient transfection assay and is also necessary for VA RNA accumulation during a virus infection.

We further show that in transient transfection experiments, the E1B-19K protein both activates basal VA RNA I transcription and overrides the effect of the repressive activity of the wt E1A on VA RNA expression (Fig. 5A). This result corroborates and extends the previous findings that E1B-19K is an activator of several RNA Pol II genes in transient transfection experiments and counteracts the inhibitory effect of E1A on RNA Pol II transcription (20, 39, 49, 66) (Fig. 5C). The mechanism by which E1B-19K participates in this process is not clear. It has been suggested that the E1B-19K protein increases gene expression by stabilizing transfected plasmid DNA (21). However, we find it unlikely that the increase in VA RNA expression by E1B-19K in our assay system is caused by a stabilization of transfected plasmid DNA since expression of β-globin mRNA in the same cells was unaffected by E1B-19K cotransfection.

The activating potential of E1B-19K for VA RNA expression was reproduced also during adenovirus infection. An adenovirus mutant unable to express the E1B-19K protein (*pm*1722) showed a reduced accumulation of VA RNA II/I (3% of wt [Fig. 6B]) although as much as one-third of wt levels of E1A mRNAs were expressed (data not shown). This was in contrast to the expression of the E4-globin reporter plasmid, which was expressed to 25% of wt mRNA levels during a *pm*1722 infection. As expected, an E1A-negative mutant (*dl*312 [Fig. 6]) was severely defective in accumulation of both RNA Pol II and III transcripts. Still, *dl*312, which has the capacity to express the E1B-19K protein, was slightly more efficient in activation of VA RNA II/I compared with *pm*1722. An important role of E1B-19K in VA RNA accumulation is also indicated from the *dl*902 infections in which VA RNA II/I is expressed to much higher levels (23% of wt [Fig. 6B]) compared with those during *dl*312 or *pm*1722 infections. Thus, our data suggest that the E1B-19K protein is essential for VA RNA transcription also during virus infections and to a lesser extent affects RNA Pol II transcription.

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