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Transcriptional Transactivation of Parvovirus B19 Promoters in Nonpermissive Human Cells by Adenovirus Type 2

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The pathogenic human parvovirus B19 contains a promoter at map unit 6 (B19p6) of the viral genome, expression from which is largely restricted to human cells in the erythroid lineage, whereas a putative promoter at map unit 44 (B19p44) is inactive during a natural viral infection. Although nonerythroid human cells, such as HeLa and KB, allow expression from the B19p6 promoter but not from the B19p44 promoter following DNA-mediated transfection, little expression from the B19p6 promoter occurs following recombinant virus infection (S. Ponnazhagan, X.-S. Wang, M. J. Woody, F. Luo, L. Y. Kang, M. L. Nallari, N. C. Munshi, S. Z. Zhou, and A. Srivastava, submitted for publication). However, significant expression from the B19p6 promoter as well as the B19p44 promoter could be detected in a human 293 cell line that expresses the adenovirus early gene products, suggesting that coinfection with adenovirus might mediate transcriptional transactivation of the B19 promoters in nonpermissive cells. Expression of the firefly luciferase reporter gene from the B19 promoters was evaluated either following plasmid transfection or following infection with the recombinant adeno-associated virus type 2 vectors. Both B19p6 and B19p44 promoters could be transactivated by coinfection with adenovirus in nonpermissive human cells, although the extent of transactivation of the B19p44 promoter was significantly lower than that of the B19p6 promoter. Expression of the adenovirus E1A proteins was necessary and sufficient for the observed transactivation of the B19 promoters. These studies further illustrate that the underlying molecular mechanisms of transactivation of parvovirus promoters in general by the adenovirus early proteins have similarities with those of the well-documented transactivation of the adeno-associated virus type 2 promoters.

Parvoviruses are among the smallest of the single-stranded DNA-containing viruses that exhibit unique replication properties (3). While the autonomous parvoviruses, such as the human pathogenic parvovirus B19 and the minute virus of mice can replicate independently (9), a nonpathogenic human parvovirus, the adeno-associated virus type 2 (AAV), requires a helper virus, such as adenovirus or herpesvirus, for its optimal replication (4). The nature of the helper functions provided by adenovirus in the AAV life cycle has been elucidated previously in some detail (26). Although B19 does not require a helper virus, the viral replication is strictly confined to the erythroid lineage in human hematopoietic cells (24, 25, 33, 34, 40, 45, 49, 55). Studies so far have established the presence of a single promoter at map unit 6 of the B19 genome, termed B19p6, that regulates transcription of both the viral nonstructural (NS-1) and the viral structural (capsid) genes (32). Although Doerig et al. (12) reported the presence of a second promoter at map unit 44 (B19p44), other investigators failed to establish its function as a promoter (20). Studies have also indicated the importance of the upstream regulatory sequences in the transcriptional control of the B19p6 promoter (5, 21). However, all previous studies have been carried out by using recombinant plasmid-mediated transfections in nonpermissive human cells that may not adequately reflect the B19 promoter functions under natural viral infection conditions (36). Similarly, a recent study using electrophoretic mobility shift assays has suggested the involvement of the transcription regulator YY-1 in the B19p6 promoter activity (23). Furthermore, since one of the AAV promoters at map unit 5 (AAVp5) contains upstream sequences that bind YY-1, which is known to exert transcriptional repression that is relieved by the adenovirus E1A protein (43), we reasoned that expression from the B19 promoters might also be modulated by the adenovirus E1A protein. The adenovirus E1A protein has been known to interact with a wide variety of transcription-regulating elements and is a potent activator of transcription, stimulating expression from both viral and cellular promoters (19).

In the present studies, we examined expression from the B19p6 promoter as well as that from the B19p44 promoter, in the context of a virus genome, by constructing recombinant AAV virions followed by infections, and compared our findings with those from plasmid DNA-mediated transfections. Here, we present a systematic analysis of the B19 promoter functions as well as the role that the adenovirus early gene product(s) plays in transactivation of these promoter sequences in nonpermissive human cells.

Human 293 cells, but not KB cells, allow expression from the B19p6 promoter following recombinant AAV-mediated infection. The details of construction of plasmids pRSVL, containing the firefly luciferase (Luc) gene (11), pSP-6, containing the B19p6 promoter-driven Luc gene in between the AAV inverted terminal repeats (ITRs) (36), and pWP-19, containing the herpesvirus thymidine kinase promoter-driven gene for resistance to neomycin (Neo^r) (31), have been described previously. A recombinant plasmid, designated pMW-5, containing the Luc gene under the control of the putative B19p44 promoter in between the AAV ITRs, was constructed as follows. First, a *Sma*I-*Tth*III fragment containing the B19p44

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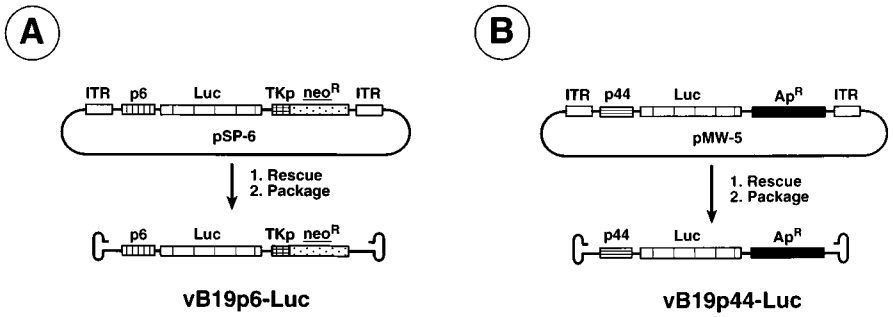

FIG. 1. Construction of the recombinant AAV B19p6-Luc and B19p44-Luc plasmids and virions. The overall strategy for construction of the recombinant plasmids and virions is described in the text. The recombinant AAV genomes were rescued from the respective plasmids and packaged separately as previously described (22, 31, 35, 39, 56–58).

sequences from plasmid pYT104v (42) was cloned at the *Sma*I site in plasmid pUC-19. The cDNA sequences of the firefly luciferase (Luc) gene from plasmid pRSVL was subsequently ligated downstream from the B19p44 promoter to generate a plasmid, designated pMW-4. This plasmid was linearized with *Xba*I and subcloned in between the AAV ITRs isolated from plasmid pWN-1 (31), to yield the plasmid pMW-5. The recombinant AAV virions containing the Luc gene under the control of either the B19p6 promoter (vB19p6-Luc; Fig. 1A) or the B19p44 promoter (vB19p44-Luc; Fig. 1B) were generated, respectively, from plasmids pSP-6 and pMW-5, essentially by following the method described previously (22, 28–31, 35–37, 39, 44). The viral titers were determined on quantitative DNA slot blots as previously described (45–48).

We have recently described that expression from the B19p6 promoter, in the context of a parvovirus genome, is restricted to human hematopoietic cells in the erythroid lineage (36, 53). However, expression from this promoter is detected in nonerythroid cells, such as HeLa, following plasmid transfections (20). Plasmid transfection and recombinant virus infection experiments were carried out under identical conditions with two additional nonerythroid human cell lines, KB (a human nasopharyngeal carcinoma cell line) and 293 (an adenovirus-transformed human embryonal kidney cell line). Plasmid DNA transfections were carried out by using the lipofectamine reagent (Gibco-BRL, Gaithersburg, Md.) as per the manufacturer's instructions. In some experiments, cells were first infected with human adenovirus type 2 (Ad2) at 37°C for 1 h; this was followed by plasmid transfections as described above. Three micrograms of each of the recombinant AAV-Luc plasmids and 1 μg of an internal control plasmid containing the cytomegalovirus promoter-driven β-galactosidase gene (pCMV-LacZ) to normalize transfection efficiencies were transfected as indicated above. In infection experiments, cells were either mock infected or infected with recombinant AAV at a multiplicity of infection (moi) of 1, either with or without coinfection with Ad2, for 1 h at 37°C. Twenty-four hours posttransfection/infection, cells were lysed and Luc activity was determined by using a Lumat LB 9501 Berthold luminometer. All transfection and infection experiments were performed at least three times, and the assays were done in duplicates.

It is evident from Fig. 2 that, in a plasmid form (pB19p6-Luc), abundant expression from the B19p6 promoter was detected both in KB (Fig. 2A) and 293 (Fig. 2B) cells, although the extent of the reporter gene activity was more pronounced in 293 cells. In addition, in view of the documented participation of the AAV ITR sequences in transcriptional transactivation of the AAV promoters (1), transfections with plasmids lacking the AAV ITRs were also carried out under identical conditions. The presence of the AAV ITRs in these plasmids had no significant effect. However, when the same construct was introduced in these cells in the form of a recombinant AAV (vB19p6-Luc), no activity from this promoter was observed in KB cells (Fig. 2A), an observation consistent with our previous studies (36). Interestingly, however, a significant level of Luc activity was observed from the same recombinant virus in 293 cells under identical conditions, suggesting that the early gene products of adenovirus that are produced constitutively in these cells might be involved in transcriptional transactivation of the B19p6 promoter.

This hypothesis was tested in experiments in which coinfection with Ad2 virions was carried out in KB and 293 cells. As shown in Fig. 3, there was a considerable increase in Luc activity following plasmid transfection in both KB (Fig. 3A) and 293 (Fig. 3B) cells in the presence of Ad2 compared with that without Ad2 (Fig. 2), and once again, the presence of the AAV ITRs in these plasmids had no significant effect. Furthermore, expression from the B19p6 promoter was detected, following recombinant AAV infection, in the presence of Ad2 in KB cells (Fig. 3A), and its extent was further augmented in 293

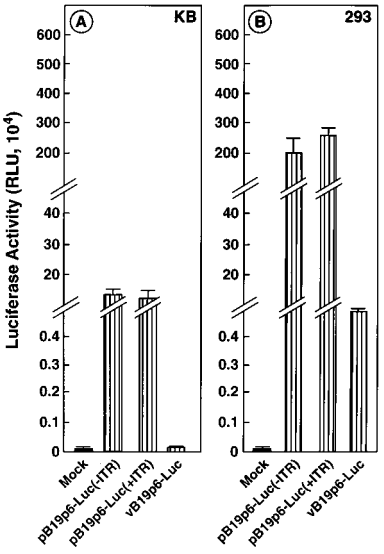

FIG. 2. Comparative analysis of expression of luciferase activity following plasmid transfection (pB19p6-Luc) and the recombinant virus infection (vB19p6-Luc) in KB (A) and 293 (B) cells. The cells were harvested 24 h posttransfection/infection, and luciferase activity was determined as described in the text. RLU, relative light units.

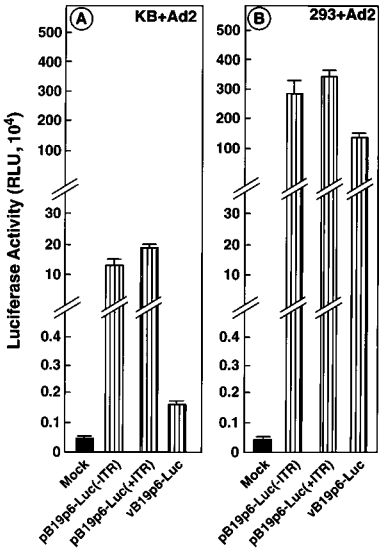

FIG. 3. Comparative analysis of expression of luciferase activity following plasmid transfection (pB19p6-Luc) and recombinant virus infection (vB19p6-Luc) in the presence of Ad2 in KB (A) and 293 (B) cells.

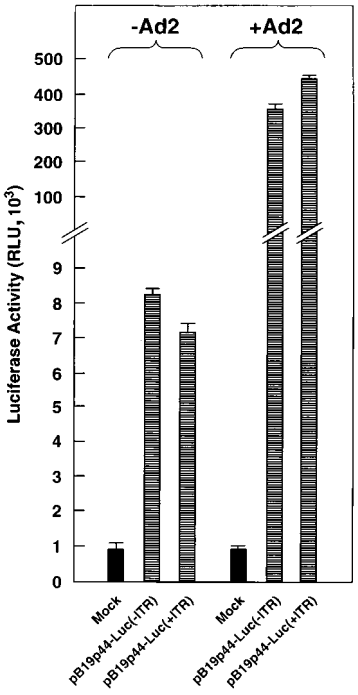

FIG. 4. Comparative analysis of expression of luciferase activity from the B19p44 promoter following transfections with plasmid pB19p44-Luc with or without the AAV ITRs and in the absence or in the presence of Ad2 infection in 293 cells.

cells in the presence of Ad2 (Fig. 3B). These data clearly indicate transcriptional transactivation of the B19p6 promoter by Ad2 early gene products. It has previously been reported that the nonstructural (NS-1) gene product of B19 can transactivate expression from the B19p6 promoter but not from the B19p44 promoter (12, 20). In our studies, we examined the effect of the NS-1 protein on expression from the B19 promoters by cotransfecting these recombinant reporter plasmids with plasmid pYT103 that contains the coding sequences of the B19 NS-1 gene (42). These experiments with KB cells, carried out either with or without coinfection with Ad2, indicated that transcriptional transactivation of the B19p6 promoter by the NS-1 protein was exceeded by that from the Ad2 early proteins and that there was a synergistic increase in the level of transactivation in the presence of the B19 NS-1 and the Ad2 early products. Similar results were obtained with 293 cells, except that the relative level of expression from the B19p6 promoter was significantly higher. Little expression from the B19p44 promoter, on the other hand, was observed in KB cells under identical conditions (see below). Whereas no significant effect of the NS-1 protein was evident, expression from the B19p44 promoter was significantly augmented in the presence of both NS-1 and the Ad2 early proteins. Again, similar results were obtained with 293 cells (data not shown).

Transcriptional transactivation of the B19p44 promoter by Ad2 early proteins. Since we were unable to detect expression from the B19p44 promoter in KB cells following plasmid-mediated transfection, we wished to examine the potential of expression from this promoter following plasmid transfections in 293 cells in the absence and in the presence of coinfection with Ad2. The results of these experiments are shown in Fig. 4. As can be seen, transcription from the B19p44 promoter, regardless of the absence or presence of the AAV ITRs, did indeed occur, and its extent was significantly enhanced in the presence of Ad2 coinfection. These data further suggested that the Ad2 early proteins were involved in transcriptional transactivation of the B19p44 promoter. These studies were extended to include transduction of KB as well as 293 cells with the recombinant vB19p44-Luc virions, in the absence or in the presence of coinfection with Ad2. The results of these experiments, presented in Fig. 5, indicate that whereas no expression from the B19p44 promoter occurred in KB cells (Fig. 5A), a significant level of expression from this promoter was detected in 293 cells (Fig. 5B). The B19p44 promoter could also be transactivated in KB cells by coinfection with Ad2 (Fig. 5A), and the extent of expression was further augmented in 293 cells

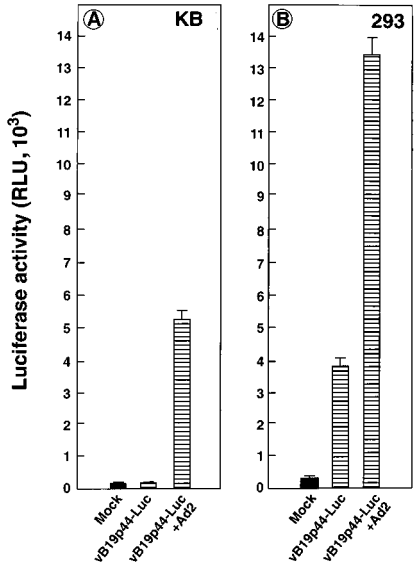

FIG. 5. Comparative analysis of B19p44-Luc activity following recombinant AAV infection in the absence (vB19p44-Luc) or in the presence (vB19p44-Luc+Ad2) of Ad2 in KB (A) and 293 (B) cells.

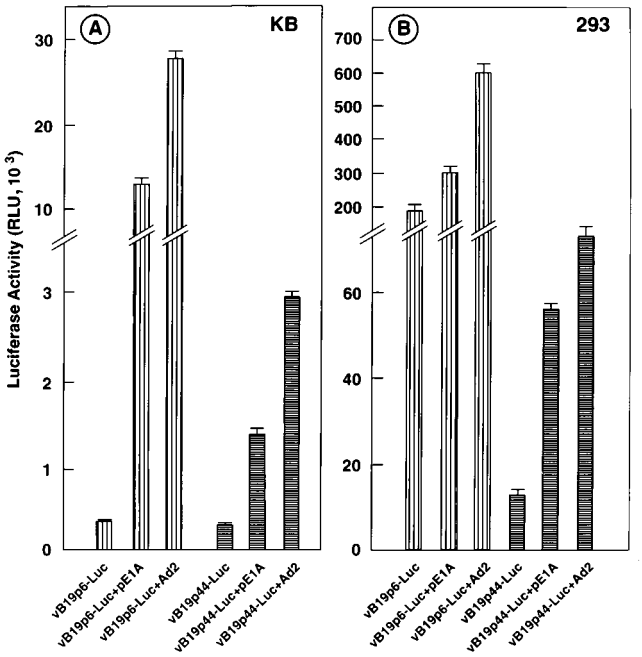

FIG. 6. Comparative analysis of expression of luciferase activity following infections with the recombinant vB19p6-Luc and the vB19p44-Luc virions in the absence and in the presence of transfection with a plasmid expressing the Ad2 E1A proteins and in the absence and in the presence of coinfection with Ad2 in KB (A) and 293 (B) cells.

(Fig. 5B). Thus, it appears that in the presence of Ad2, the B19p44 promoter sequences constitute a functional unit.

Ad2 E1A proteins mediate transcriptional transactivation of the B19 promoters. Although these studies implicated the Ad2 early proteins in mediating the transcriptional transactivation of the B19 promoters, it was next of interest to examine this possibility directly. KB and 293 cells were transfected with a recombinant plasmid that constitutively expresses the Ad2 E1A proteins; this was followed by infections with the recombinant vB19p6-Luc and the vB19p44-Luc virions under identical conditions. The results of these experiments are depicted in Fig. 6. It is interesting to note that whereas little expression from the B19p6 and the B19p44 promoters occurred in KB cells (Fig. 6A), the Ad2 E1A proteins alone were sufficient to cause substantial transactivation of these promoters. There was approximately a further twofold increase in the presence of Ad2 coinfection. A similar pattern was obtained with 293 cells (Fig. 6B), although expression from these promoters in the absence of transfection with the E1A plasmid or coinfection with Ad2 was also detected, as observed before. These data strongly suggest that the Ad2 E1A proteins are necessary and sufficient, for the most part, to induce expression from the B19 promoters in nonpermissive human cells, although the involvement of other adenovirus gene products in the observed transcriptional transactivation of the B19 promoters could not be ruled out. Similarly, our studies did not address the less likely possibility that the adenovirus gene products stimulated the entry, the uncoating of the viral DNA, and the synthesis of double-stranded DNA templates, leading to the observed transcriptional transactivation of the B19 promoters.

The regulation of gene expression of the pathogenic human parvovirus B19 is unique in that, unlike all other known parvoviruses, expression of the viral nonstructural and structural genes is under the control of a single promoter (B19p6). Although a putative promoter-like sequence is present at roughly the same map unit (B19p44), as in other parvovirus genomes, there are conflicting data with regard to the functional activity of this promoter (12, 20). Since all previous studies on B19 promoter functions have been carried out with DNA-mediated transfection using different methods (electroporation, calcium phosphate, and DEAE-dextran), we have reevaluated this issue by using a physiologically more relevant system of infection with a recombinant parvovirus. Interestingly, we have thus far failed to obtain expression from the B19p6 promoter, in the context of a parvovirus genome, in established nonerythroid (HeLa and KB) as well as erythroid (K562) cell lines known to be nonpermissive for B19 replication (33), whereas abundant expression from this promoter has been documented by us and others following plasmid transfection in these cell lines (20, 36). In the course of these studies, we noted, however, that expression from the B19p6 promoter could be readily detected in the vB19p6-Luc virus-infected 293 cells, suggesting that coinfection with Ad2 might transactivate B19 promoters in these otherwise nonpermissive cells. It is well established that the adenovirus E1A protein is a prototype promiscuous transcriptional transactivator (17). A substantial body of evidence indicates that E1A targets a promoter by interacting with the DNA-binding domain of cellular transcription factors, and the activation regions of E1A and the cellular proteins then cooperate to stimulate transcription (19). Two distinct classes of factors, the TATA box binding protein and the activating transcription family (ATF), have recently been shown to directly interact with the transactivation domain of E1A (17, 19). E1A also interacts with diverse DNA-binding domains, including those of AP-1, SP-1, and USF (6, 10, 13, 15, 16, 18, 50, 52), although the binding with SP-1 and USF is not as strong as that with ATF members or AP-1. The E1A protein has also been known to play a vital role in AAV replication (7, 51). Other virus promoters that respond to E1A protein transactivation include those of the herpes simplex virus type 1 gpD and thymidine kinase genes (13, 54), the bovine papillomavirus type 1 LCR (2), the human cytomegalovirus 1E gene (14), and the long terminal repeats of human T-cell leukemia virus type 1 (HTLV-1) and -2, human immunodeficiency virus, and Rous sarcoma virus (8, 27, 38). In AAV, E1A has been shown to transactivate both AAVp5 and AAVp19 promoters that regulate the expression of the viral nonstructural *rep* gene (4, 7, 51).

The B19p6 promoter is characterized by the presence of various *cis*-acting elements that could potentially play a vital role in regulating the overall transcription rate (Fig. 7). There are three binding sites for factor SP-1 (5, 21), and the importance of this sequence in in vitro transcription has previously been documented (5). SP-1 binding sites have also been known to be induced by the E1A protein (16). There is also an ATF-2-like sequence present in upstream juxtaposition to the TATA box. E1A has been known to directly bind to ATF members and TATA box binding protein (17, 19). Recent studies have identified binding sites for transcription factor YY-1 in the B19p6 promoter (23). YY-1, in the presence of an SP-1 binding site(s), has been known to exert a synergistic enhancement of the level of transcription of the AAVp5 promoter in the presence of E1A proteins (41). However, in AAV, YY-1 acts as a repressor of transcription from the AAVp5 promoter which is relieved by E1A (7, 43). The presence of several of these elements in the B19p6 promoter, therefore, accounts for its inducibility by E1A.

It is interesting to note that, in our experiments, the B19p44 promoter could also be transactivated by adenovirus early gene products. Previous studies have led to contradicting views on the authenticity of the B19p44 as a promoter. Whereas Doerig

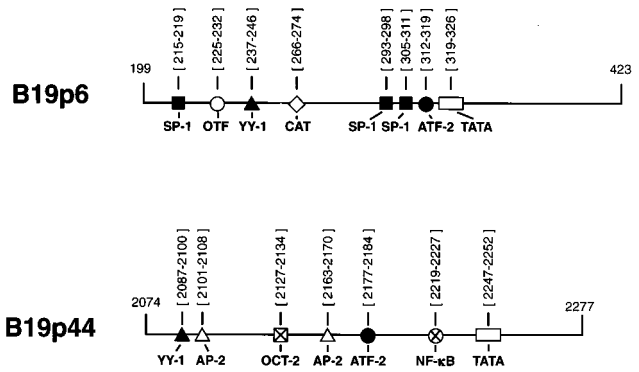

FIG. 7. Schematic representation of the B19p6 and B19p44 promoter regions used in the present investigation. The potential *cis*-acting elements and their relative locations in the promoter region correspond to the sequence published by Shade et al. (42). The closed symbols indicate the binding sites for Ad2-responsive factors.

et al. (12) reported expression from the B19p44 promoter, Liu et al. (20) failed to observe any activity from this promoter. The presence of various consensus *cis*-acting elements upstream of this promoter (Fig. 7), specifically those that respond to E1A, suggests a possible functional significance. For example, the ATF-2 sequences and the TATA-box-like sequences in this promoter could play a vital role in transcriptional transactivation. This promoter also contains an upstream sequence resembling the YY-1 binding site. Whether this site is utilized in E1A-mediated release of transcriptional repression, similar to that described for the AAVp5 promoter, remains to be determined. Similarly, although the B19 NS-1 protein has been shown to transactivate the B19p6 promoter but not the B19p44 promoter (12) in the presence of both B19 NS-1 and adenovirus early proteins, there was a considerable enhancement of B19p44 promoter activity.

Although the significance of the observed transactivation of B19 promoters by adenovirus E1A proteins in the life cycle of parvovirus B19 remains to be explored, given the remarkable erythroid cell tropism of B19, the potential susceptibility of human vascular endothelial cells to adenovirus as well as B19 raises the possibility of interactions between these two groups of viruses. The adenovirus-mediated transactivation of the B19 promoters thus parallels the well-documented transactivation of the AAV promoters by the E1A proteins. At the very least, the studies reported here illustrate the need to search for the putative intracellular erythroid transcription factor(s) that exerts a remarkable control on expression from the B19 promoters in primary human hematopoietic progenitor cells, since the binding sites for any of the known erythroid cell-specific transcription factors are not readily apparent in these promoter sequences.

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