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Construction of a recombinant human parvovirus B19: Adeno-associated virus 2 (AAV) DNA inverted terminal repeats are functional in an AAV-B19 hybrid virus

(palindromes/DNA replication/viral vectors/erythropoiesis)

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ABSTRACT **To facilitate genetic analysis of the human pathogenic parvovirus B19, we constructed a hybrid B19 viral genome in which the defective B19 inverted terminal repeats were replaced with the full-length inverted terminal repeats from a nonpathogenic human parvovirus, the adeno-associated virus 2 (AAV). The hybrid AAV-B19 genome was rescued from a recombinant plasmid and then the DNA was replicated upon transfection into adenovirus 2-infected human KB cells in the presence of AAV genes coding for proteins required for AAV DNA replication (AAV-Rep proteins). In addition, in the presence of AAV genes coding for the viral capsid proteins (AAV-Cap proteins), the rescued/replicated hybrid AAV-B19 genomes were packaged into mature AAV progeny virions, which were subsequently released into culture supernatants. The recombinant AAV-B19 progeny virions were infectious for normal human bone marrow cells and strongly suppressed erythropoiesis *in vitro*. The availability of an infectious recombinant B19 virus should facilitate the mutational analysis of the viral genome, which, in turn, may yield information on individual viral gene functions in B19-induced pathogenesis. The hybrid AAV-B19 genome may also prove to be a useful vector for gene transfer in human bone marrow cells.**

Parvoviruses are among the smallest of the DNA-containing viruses that infect a wide variety of vertebrates (1–4). Two parvoviruses of human origin, the adeno-associated virus 2 (AAV) and the parvovirus B19, have been extensively studied (5–10). Whereas AAV causes no known disease (11), B19 has been shown to be the etiologic agent of various clinical disorders in humans, including transient aplastic crises associated with various hemolytic anemias (12, 13), erythema infectiosum (a common childhood rash), also called the "fifth disease" (14), polyarthralgia syndrome (15, 16), hydrops fetalis (17, 18), and chronic bone marrow failure (19). AAV requires coinfection with a helper virus, either adenovirus (20), herpesvirus (21), or vaccinia virus (22). B19, on the other hand, is an autonomously replicating parvovirus but has so far been shown to replicate only in erythroid progenitor cells in human bone marrow (23–25). Both AAV and B19 contain a linear, single-stranded DNA genome but DNA strands of both polarities are encapsidated in separate, mature progeny virions (26, 27). Both genomes have been molecularly cloned (27–29) and their nucleotide sequences have been determined (30–32).

The AAV genome contains inverted terminal repeats (ITRs) of 145 nucleotides, 125 nucleotides of which form a palindromic hairpin that plays a crucial role during AAV DNA replication (30, 33) as well as during rescue from a recombinant plasmid (28, 34). The cloned AAV genome is thus infectious (28, 29, 35). The B19 genome also contains ITRs of ≈300 nucleotides, but the cloned B19 genome is not infectious, most likely because of large deletions introduced in its ITRs, during the cloning procedure (27, 32).

Despite significant advances in understanding the molecular biology of the parvovirus B19 in the recent past (23–25, 36–40), studies on its genetic analysis have been hampered due to unavailability of an infectious B19 clone. We, therefore, constructed a hybrid AAV-B19 genome that can be packaged into mature AAV virions in KB cells and that is infectious for normal human bone marrow cells (NBM cells) *in vitro*. This report describes the construction and characterization of this recombinant infectious parvovirus B19.

MATERIALS AND METHODS

Viruses and Cells. Human AAV (AAV-2H) and parvovirus B19 were provided by K. I. Berns (Cornell University Medical College, New York) and N. S. Young (National Heart, Lung, and Blood Institute, Bethesda, MD), respectively. Human adenovirus 2 (Ad2) was obtained from K. H. Fife (Indiana University School of Medicine). AAV was propagated in human KB cells in the presence of Ad2 in Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum essentially as described (31, 33). NBM cells were obtained from healthy volunteer donors with informed consent.

Plasmids and DNAs. The cloned B19 plasmids pYT103 and pYT107 (27) were a gift from P. Tattersall (Yale University, New Haven, CT). A plasmid containing the modified AAV genome (psub201) has recently been described (35). The plasmid pAAV/Ad containing the entire coding sequence of AAV has been described elsewhere (41).

Construction of an AAV-B19 Recombinant Plasmid. The cloned B19 DNA pYT103 (27) contains a frame-shift mutation at nucleotide position 3940 (32). This mutation was corrected by replacing a 3606-base-pair (bp) *Xba* I–*Kpn* I fragment of pYT103 with a similar *Xba* I–*Kpn* I fragment isolated from plasmid pYT107, which does not contain this mutation. All constructions were carried out by using standard procedures (42). Briefly, the AAV-containing plasmid psub201 (35) was digested to completion with *Xba* I and the plasmid vector

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Abbreviations: AAV, adeno-associated virus 2; Ad2, adenovirus 2; ITR, inverted terminal repeat; NBM cells, normal human bone marrow cells; Epo, erythropoietin; PHALCM, phytohemagglutinin-activated L-cell-conditioned medium; CFU-GM, colony-forming units-granulocyte/macrophage; BFU-E, burst-forming units-erythroid; CFU-E, colony-forming units-erythroid.

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DNA containing the AAV ITR was purified from agarose gels as described (43). Similarly, the B19 clone pYT103 (27) was partially digested with *Dra* I. This restriction endonuclease cleaves the cloned B19 DNA at 10 sites (32). The B19 insert lacking the partially deleted ITR was isolated and used in subsequent cloning as follows. *Xba* I–*Eco*RI–*Xba* I adaptors were added to the vector DNA, and *Eco*RI linkers were ligated to the B19 insert DNA. Following digestion with *Eco*RI, the vector and insert DNAs were ligated using T4 DNA ligase and were used in transformation of competent *Escherichia coli* HB101 cells (42). Recombinant plasmids containing the AAV ITRs flanking the B19 sequences were identified, and one clone (pAS313) was chosen for further studies. All experiments involving recombinant plasmids and viruses were carried out under BL-2 containment limits as specified by the National Institutes of Health.

DNA Rescue/Replication Assays. Transfections were carried out by the DEAE-dextran method (44) using 1.0 μg each of purified plasmid DNAs per ml with 70% confluent KB monolayer cultures in 100-mm dishes. Unless otherwise stated, the transfection mixture also contained Ad2 at a multiplicity of infection of 10 (34, 45). Low molecular weight DNA was isolated at various times after transfection/infection by the method described by Hirt (46), digested with *Dpn* I or other restriction endonucleases, and analyzed on Southern blots (47) by using a ^{32}P-labeled B19-insert DNA probe radiolabeled to specific activity $>1 \times 10^9$ cpm/μg of DNA by the random hexanucleotide primer method described by Feinberg and Vogelstein (48). Southern blots were hybridized (0.75 M NaCl/75 mM sodium citrate/50% formamide, 42°C), washed under stringent conditions (15 mM NaCl/1.5 mM sodium citrate, 65°C), and autoradiographed at room temperature for 1–2 hr.

Progeny Virus Release Assays. Culture supernatants from KB cells were collected 48–72 hr after transfection/infection, centrifuged at 13,000 × *g* for 5 min to remove cell debris, denatured with NaOH at a final concentration of 0.5 M, and heated at 60°C for 1 hr to disrupt virions, denature DNAs, and degrade RNAs. Following neutralization with 3 M NaCl/0.3 M sodium citrate, 2-fold serial dilutions of equivalent amounts were filtered through a DNA dot blot apparatus and analyzed with the B19 probe as described above (23–25). In some experiments, total cell lysates were also included in this assay.

Progeny virions were isolated as follows: 72 hr after transfection/infection, cells from 8–10 dishes were scraped, pooled, and pelleted. Cell pellets were washed with phosphate-buffered saline (PBS), resuspended in fresh medium, and frozen and thawed three times to disrupt the cells. Cell lysates were heated at 56°C for 1 hr to inactivate Ad2 virions and used directly or filtered through 0.45-μm filters and stored frozen in small aliquots at −80°C (49).

Infection of NBM Cells *in Vitro* and Hematopoietic Colony Assays. NBM cells were fractionated on Ficoll/Hypaque density gradients (specific gravity = 1.077 g/cm^3) and nonadherent mononuclear cells were isolated essentially as described (25). NBM cells (1×10^7) were incubated with the authentic B19 parvovirus and the AAV-B19 recombinant virus separately at 4°C for 2 hr. Cells were washed twice with PBS to remove the virus inoculum and resuspended in Iscove's modified Dulbecco's medium containing 20% fetal calf serum and 1 unit of erythropoietin (Epo) per ml as described (23–25). Cells were incubated at 37°C in a CO_2 incubator, and low molecular weight DNA was isolated and analyzed on Southern blots as described above.

Uninfected and infected NBM cells were also used in *in vitro* colony assays. Colony formation by myeloid and erythroid progenitor cells was performed as described (25). Colony-forming unit-erythroid (CFU-E) colonies were scored on day 7, and burst-forming unit-erythroid (BFU-E) clusters and colony-forming unit-granulocyte/macrophage (CFU-GM) colonies were scored from the same dishes on day 14.

RESULTS

Construction and Characterization of the AAV-B19 Recombinant Genome. The original B19 DNA clone pYT103 (27) contains a frame-shift mutation at nucleotide position 3940 (32). This mutation was corrected to generate the pYT103c clone as described under *Materials and Methods*. The general overall strategy we used to construct the AAV-B19 recombinant clone pAS313 is presented in Fig. 1, and the

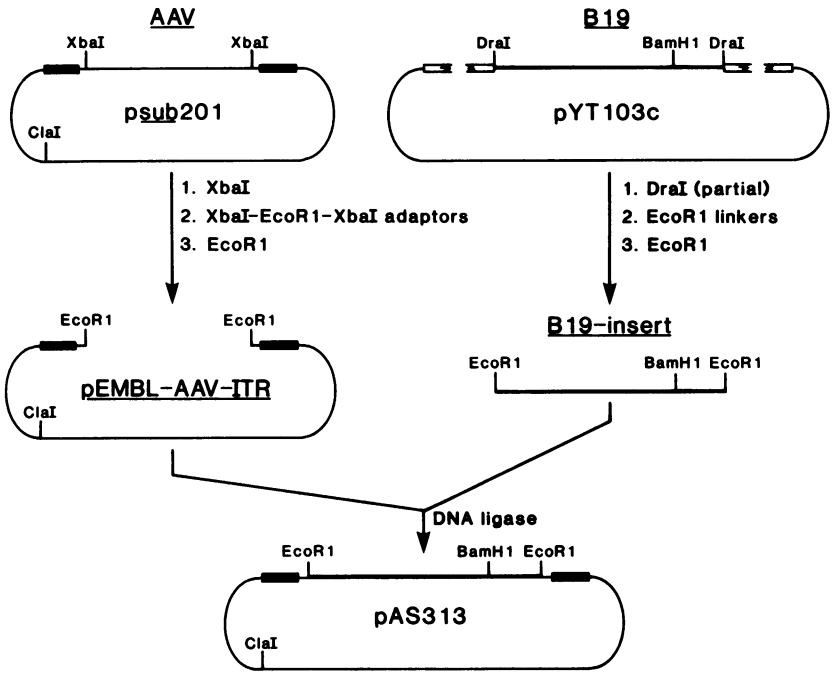

FIG. 1. General overall strategy for the construction of plasmid pAS313 containing an AAV-B19 recombinant genome. Each experimental step is detailed in the text. Closed and open (broken) boxes denote the AAV and B19 ITR sequences, respectively.

details of this construction are provided under *Materials and Methods*.

Previous studies have established that the AAV genome can be rescued from transfected recombinant plasmids and then the viral DNA can be replicated in human cells in the presence of adenovirus (28, 29, 34, 35, 50). The rescue/replication is mediated by the AAV ITRs. We, therefore, exploited the rescuability feature of the AAV ITRs in the AAV-B19 recombinant genome (pAS313). In preliminary experiments, however, it became evident that the AAV-B19 recombinant genome could not be rescued in human KB cells under the conditions where the wild-type AAV genome undergoes successful rescue/replication (see below). In addition to coinfection with adenovirus, transfection with a recombinant plasmid, pAAV/Ad (41), which encodes all of the AAV proteins but which does not undergo rescue because the ITRs have been deleted from this construct, was also required (35). Plasmids pAS313 and pAAV/Ad were cotransfected into Ad2-infected KB cells, and low molecular weight DNA samples isolated at various times after transfection were digested with *Dpn* I to degrade unreplicated input plasmid DNAs and analyzed on a Southern blot using a ^{32}P-labeled B19-insert DNA probe. Results are shown in Fig. 2. It is evident (Fig. 2*A*) that the AAV-B19 recombinant genome was rescued from the plasmid pAS313, which then underwent DNA replication in KB cells to generate the characteristic monomeric and dimeric forms of parvoviral replicative DNA intermediates by 48 hr (lane 2), the extent of which was augmented by 72 hr (lane 3). Data in Fig. 2*B* document that no rescue/replication occurred in the absence or presence of Ad2 and in the absence of pAAV/Ad (lanes 1 and 2) or in the presence of pAAV/Ad but in the absence of Ad2 (lane 3). Thus, the presence of Ad2 and the complementing plasmid pAAV/Ad was required for efficient rescue/replication of the AAV-B19 recombinant genome from pAS313 (lane 4). When pAS313 plasmid DNA was linearized by digestion with *Bam*HI (a single site in the B19 sequence; Fig. 1) prior to transfection, no rescue/replication occurred even in the presence of Ad2 and pAAV/Ad (lane 5), whereas prior digestion of the pAS313 DNA with *Cla* I (a single site in the plasmid vector) had no significant effect as expected (35) on the rescue/replication of the AAV-B19 recombinant genome (lane 6). The apparent differences in the hybridization intensities reflect different amounts of the input DNA.

Packaging of the AAV-B19 Recombinant Genomes into AAV Particles. The rescued/replicated AAV DNA strands, upon synthesis of the viral capsid proteins, have been shown to be packaged into mature progeny AAV virions that are released into the culture supernatants (45). It was, therefore, of significant interest to examine whether the AAV-B19 recombinant genomes could also be packaged into mature, progeny AAV virions and released. Culture supernatants from Ad2-infected KB cells also transfected with pAS313 and/or pAAV/Ad plasmids were collected 48 hr after transfection/infection and analyzed on a quantitative DNA dot blot and probed with a B19 DNA probe as described under *Materials and Methods*. Results of such an experiment are shown in Fig. 3. As can be seen in Fig. 3*A*, no hybridization signal was detected in culture supernatants from Ad2-infected KB cells without plasmid transfections (row 1), transfection with plasmid pAS313 (row 2), or transfection with plasmid pAAV/Ad alone (row 3). Cotransfection of plasmids pAS313 and pAAV/Ad and infection with wild-type Ad2 were required to detect the release of mature progeny virions (row 4). Also, when Ad2-infected KB cells were cotransfected with pAAV/Ad and *Bam*HI-cleaved pAS313, no virus release was detected in the culture supernatants, whereas *Cla* I digestion of pAS313 DNA had no effect (data not shown). These results thus corroborate the rescue/replication data presented in Fig. 2. Data in Fig. 3*B* document the time-dependent accumulation of intracellular AAV-B19 recombinant progeny virion DNA (rows 1–3). We also included on the same blot 2-fold serial dilutions of a known amount (10^3 pg) of the B19-insert DNA from plasmid pYT103 (row 4), which served as a positive control for hybridization and was useful in determining the AAV-B19 DNA copy number per cell (23–25). Based on the comparison of hybridization intensities, we estimate that the average recombinant viral DNA copy number in KB cells 72 hr after transfection/infection was $\approx 1 \times 10^9$ per cell.

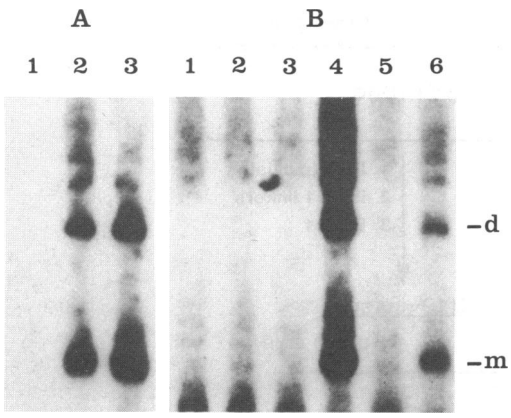

FIG. 2. Southern blot analysis of rescue/replication of the AAV-B19 recombinant genome from plasmid pAS313 in human KB cells. (*A*) Kinetics of rescue/replication in the presence of adenovirus and the AAV helper plasmid pAAV/Ad (lane 1, 24 hr; lane 2, 48 hr; lane 3, 72 hr). (*B*) Rescue/replication of the AAV-B19 genome in the absence of Ad2 (lane 1); in the presence of Ad2 but in the absence of pAAV/Ad (lane 2); in the presence of pAAV/Ad but in the absence of Ad2 (lane 3); in the presence of Ad2 and pAAV/Ad (lane 4); prior digestion of plasmid pAS313 with *Bam*HI (lane 5) or with *Cla* I (lane 6) and rescue/replication assays in the presence of Ad2 and pAAV/Ad. m and d, monomeric and dimeric forms of the replicative DNA intermediates, respectively.

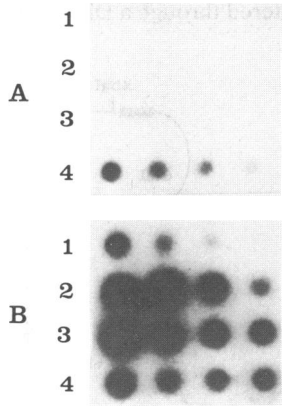

FIG. 3. DNA dot blot analysis of 2-fold serial dilutions of KB culture supernatants and cell lysates for the accumulation and release of the progeny AAV-B19 recombinant virions. (*A*) Dependence of assembly and release of virions containing the rescued/replicated AAV-B19 genomes on Ad2 and pAAV/Ad. No viral DNA-specific hybridization is detected in mock-transfected, Ad2-infected KB cells (row 1) or Ad2-infected cells transfected with either pAS313 (row 2) or pAAV/Ad (row 3) alone. Ad2, pAS313, and pAAV/Ad are required for virus assembly and release (row 4). (*B*) Kinetics of intracellular accumulation of the AAV-B19 recombinant virion DNA in KB cells (row 1, 24 hr; row 2, 48 hr; row 3, 72 hr after transfection/infection). Two-fold serial dilutions of 10^3 pg of B19 insert DNA (row 4) were also included in this panel to serve as a positive control for hybridization and to calculate the AAV-B19 DNA copy number per cell.

Biological Activity of the AAV-B19 Recombinant Virus in NBM Cells. Wild-type AAV virions adsorb to and enter bone marrow cells but no subsequent replication occurs either in the presence or absence of Ad2 (A.S., unpublished observations). We examined whether the progeny AAV-B19 recombinant virions released from KB cells were infectious for human bone marrow cells. Equivalent numbers of low-density normal bone marrow mononuclear cells were infected with the authentic B19 virions and the AAV-B19 recombinant virions separately under identical conditions in the presence of either Epo or phytohemagglutinin-activated L-cell-conditioned medium (PHALCM) as described under *Materials and Methods*. Low molecular weight DNA isolated from infected cells was analyzed on a Southern blot and probed with a B19 DNA probe as described above. The results of these experiments are shown in Fig. 4. It is interesting to note that the AAV-B19 recombinant virions underwent productive infection in human bone marrow cells, as determined by production of the characteristic monomeric and dimeric replicative DNA intermediates, to nearly the same extent as that of the authentic B19 virions in the presence of Epo (lane 1 vs. lane 2). Such an Epo dependence of B19 replication in human bone marrow erythroid cells has been documented before (23, 24). Low-level replication occurred with both virions in the presence of PHALCM (lanes 3 and 4). The significance of the hybridization signal near the dimeric replicative intermediate (lanes 2 and 4) is not clear. It may represent either the residual input plasmid DNA or an aberrant form of replicative DNA intermediate. Experiments are necessary to distinguish between these two possibilities. These results, nonetheless, document that the AAV-B19 recombinant virions were indeed infectious for human bone marrow cells, and their similar growth requirements further suggest that their replication was restricted to cells in the erythroid lineage.

We further evaluated the influence of the AAV-B19 recombinant virions on normal human hematopoiesis *in vitro* by colony assays. As has been observed with the authentic B19 parvovirus (10, 25), the AAV-B19 recombinant parvovirus also strongly suppressed BFU-E and CFU-E colony formation, whereas the hybrid virus had no significant effect on CFU-GM colony formation (Table 1). These data further document the remarkable tissue tropism of the B19 parvovirus for human hematopoietic cells of erythroid lineage (23–25).

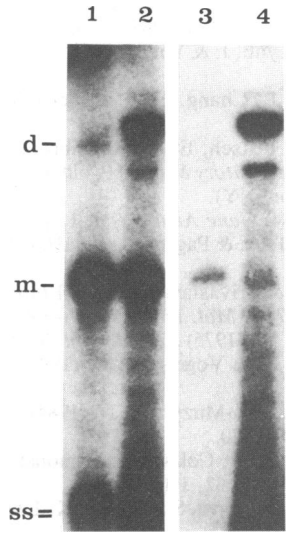

FIG. 4. Southern blot analysis for replication of the authentic B19 and the AAV-B19 recombinant virions in NBM cells. Low molecular weight DNA samples isolated from B19-infected (lanes 1 and 3) and AAV-B19-infected (lanes 2 and 4) NBM mononuclear cells grown in the presence of either Epo at 1 unit/ml (lanes 1 and 2) or 10% PHALCM (lanes 3 and 4) were analyzed. d, m, and ss denote the dimeric and monomeric forms of the replicative DNA intermediates and the single-stranded progeny viral DNA, respectively.

Table 1. Effect of parvovirus B19 and the AAV-B19 recombinant parvovirus on colony formation by normal human hematopoietic progenitor cells

Infection of NBM cells	Number of colonies or clusters		
	BFU-E	CFU-E	CFU-GM
Mock	82 ± 2	237 ± 36	30 ± 2
B19	19 ± 2 (−77)*	143 ± 7 (−40)†	31 ± 3 (+3)
AAV-B19	26 ± 6 (−68)‡	148 ± 11 (−38)†	30 ± 1 (0)

Low-density bone marrow mononuclear cells were either mock-infected or infected with B19 or AAV-B19 and incubated at 37°C for 48 hr prior to assay in triplicate for colony formation in the presence of 10% 5637-conditioned medium for CFU-GM and 5% 5637-conditioned medium and 1 unit of Epo for BFU-E and CFU-E. Results are expressed as mean numbers of colonies or colonies plus clusters ± 1 SEM. Values in parentheses represent the percent significant change from the mock-infected control.
*$P < 0.0001$.
†$P < 0.05$.
‡$P < 0.001$.

DISCUSSION

We have constructed an AAV-B19 hybrid parvovirus that is infectious and biologically indistinguishable from the authentic parvovirus B19 in *in vitro* studies. The availability of an infectious B19 genome overcomes the obvious problem to create genetic mutations in the viral genome and to examine the role of individual viral gene functions in B19-induced pathogenesis.

The biological activity of the AAV-B19 recombinant parvovirus is of significance in several respects. First, it should be noted that although AAV and B19 contain linear, single-stranded DNA genomes, their genomes share no homology at the nucleotide sequence level (27). Interestingly, however, AAV and B19 DNAs contain ITRs, a feature that distinguishes these viruses from other autonomously replicating mammalian parvoviruses whose termini are not present as inverted repeats (3–7). All known parvoviral genomes, nonetheless, contain terminal palindromic structures that play a crucial role in their DNA replication (4, 7). In view of the previous studies on the role of palindromic termini in AAV DNA replication, it has become abundantly clear that, for the most part, the hairpin structure is of much more critical importance than the nucleotide sequence *per se* (51, 52). Since AAV and B19 DNA termini would be expected to form very similar hairpin structures, it is perhaps not surprising that the AAV hairpin termini are functional in the AAV-B19 recombinant genome. Mechanistically, however, it is intriguing that the proteins involved in parvoviral DNA replication (Rep proteins) for AAV and B19 also appear to share strong functional similarities. AAV encodes two major Rep proteins, to date only one of which has been demonstrated to be required for viral growth (7, 31), whereas B19 has so far been shown to code for only one nonstructural protein, which may be the Rep protein (32, 37). The replication of the AAV-B19 recombinant genome in human KB cells, where only AAV Rep proteins are expressed (from plasmid pAAV/Ad), and in human bone marrow cells, where only B19 Rep proteins are expressed (from the AAV-B19 recombinant genome), suggests functional similarities between the AAV and B19 Rep proteins. Our recent studies in which wild-type B19 DNA replication could be catalyzed in human KB cells in the presence of AAV Rep proteins have indeed substantiated these observations (A.S., unpublished data). These data lend

strong support to a unified molecular mechanism underlying replication of linear, single-stranded DNA molecules (53).

A second useful feature of the AAV-B19 recombinant virus is its possible use as a vector in gene transfer studies with human bone marrow cells. In addition to the high efficiency, the AAV-B19 recombinant virus-mediated gene transfer may also prove advantageous over the more commonly used retroviral long terminal repeat (LTR)-based vectors primarily because of its selective tissue tropism for the human bone marrow progenitor cells. Furthermore, the expected stable integration of foreign genes mediated by AAV ITRs in the absence of DNA replication without alterations in the target cell phenotype (54) may offer added safety features in contrast to the retroviral LTR-based vectors. Since AAV ITRs are also known to mediate the rescue of the integrated viral genome from the host cell chromosome in the presence of adenovirus (28, 29, 34, 35), the potential for excision and removal of the integrated foreign gene remains an interesting possibility.

And finally, despite the ubiquitous nature of parvoviruses in general (3–7), B19 has proven to be an extremely fastidious virus with regard to its rigid growth requirements and narrow host range (23–25). With the availability of a recombinant infectious clone, the molecular mechanisms underlying the remarkable tissue tropism of parvovirus B19 may become amenable to experimental analysis.

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