Adeno-associated virus type 2-mediated inhibition of human immunodeficiency virus type 1 (HIV-1) replication: involvement of p78rep/p68rep and the HIV-1 long terminal repeat

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Microinjection of wild-type adeno-associated virus type 2 (AAV-2) DNA and infectious human immunodeficiency virus type 1 (HIV-1) proviral DNA into the nuclei of human epithelioid SW480 cells leads to specific inhibition of HIV-1 replication. Mutational analysis of the AAV genome showed that this negative interference can be assigned to a functional AAV-2 rep gene. Moreover, the p78rep/p68rep proteins are sufficient for the anti-HIV-1 effects. The rep gene also inhibits the expression of a chloramphenicol acetyltransferase (CAT) gene driven by the U3/R portion of the HIV-1 long terminal repeat (LTR) in the absence of tat expression. This suggests that the U3/R portion of HIV-1 contains elements responsible for the AAV-2 rep-mediated inhibition of HIV-1 LTR-driven CAT gene expression and, probably, also of HIV-1 replication. The results add support for the general significance of AAV-2 and specifically the rep gene as tools for down-regulating heterologous gene expression.
transported into the nucleus after transfection of rep+ mutants, but not rep− mutants (Fig. 1c, d). Since the rep− and rep+ constructs differ by only four additional bp causing a frameshift within the coding region of rep, the potential binding properties of these sequences should not be affected. However, there is a clear correlation between HIV-1 inhibition and the presence or absence of rep proteins.

Since the rep+, cap− mutant (pTAV2-6), which has been shown to be defective in AAV-2 production (Heilbronn et al., 1990), has an anti-HIV-1 efficacy (99% inhibition) very similar to that of wild-type AAV pTAV2 (rep+, cap+; 97% inhibition), the inhibition of HIV-1 replication must occur in microinjected SW480 cells. This means that interference of AAV-2 particles with HIV-1 replication during amplification of progeny HIV-1 in co-cultured MT-4 cells cannot be the cause of the AAV-mediated repression of HIV-1 replication.

To exclude the possibility that the rep gene-mediated inhibition of HIV-1 is a result of cell toxicity induced by the rep proteins, resulting in inability of these cells to replicate HIV-1, we tested the ability of the rep-expressing cells to co-express unrelated genes. Human SW480 cells were microinjected with a Rous sarcoma virus long terminal repeat (LTR)-driven β-galactosidase gene, or a human cytomegalovirus (HCMV) immediate early (IE) promoter/enhancer-driven chloramphenicol acetyltransferase (CAT) gene and rep+ mutants (pTAV2 or pTAV2-6) or rep− mutants (pTAV2-3 or pTAV2-8). CAT or β-galactosidase expression was measured 24 h after microinjection as described (Rittner et al., 1991). Expression of the rep gene in co-injected cells did not reduce the percentage of cells expressing the β-galactosidase gene or the level of CAT expression (data not shown). This indicates that SW480 cells permit gene expression from two heterologous promoters in the presence of the rep gene. Transient rep gene expression has been shown not to influence gene expression from the HCMV or simian virus 40 promoter in hamster or human cells (Beaton et al., 1989; Heilbronn et al., 1990). Therefore, we assume that microinjected cells retain the potential for expression of both AAV-2 and HIV-1 genes, including subsequent HIV-1 production. Thus, the inhibition of HIV-1 replication is due to specific anti-HIV-1 functions of the AAV-2 rep gene.

Four different proteins are expressed from the rep open reading frame by differential use of the AAV-2 p5 and p19 promoters, as well as by differential splicing. To test the role of individual rep gene products we constructed a series of mutants according to the protocol of Taylor et al. (1985). These constructs were expressed under the control of the HCMV IE enhancer/promoter in plasmid pKEX (Rittner et al., 1991) and co-injected with cloned HIV-1 DNA. The expression of p78rep/p68rep from plasmid construct M1 (Fig. 2b) was sufficient to mediate inhibitory effects on HIV-1 LTR-driven gene expression, whereas the M25 construct, expressing p52rep/p40rep, clearly demonstrated that the two small rep proteins are not inhibitory. To exclude the possibility...
that p52rep/p40rep play an additional role in the presence of p78rep/p68rep, the mutant Met225-Pro225 was generated (Fig. 2b); this mutant does not express p52rep/p40rep. This mutant completely inhibited HIV-1 LTR-driven gene expression. This suggests that the N-terminal portion unique to the p78rep/p68rep proteins is necessary for the inhibition process. To dissect the peptide sequence elements of the N terminus of p78rep/p68rep required for rep-mediated inhibition of HIV-1 replication further, we generated a mutant rep protein which starts at methionine 174 (M174; Fig. 2). This truncated form, which is normally expressed and translocated to the nucleus, produced significant inhibition of HIV-1 replication (70% compared to 84% for p78rep/p68rep), whereas M174 is not capable of producing AAV replication (J. Klein-schmidt, F. Weindler & R. Heilbronn, unpublished results). However, this result implies that the 51 amino acids at the N terminus of M174 not in p52rep/p40rep possibly contain elements involved in the rep-mediated inhibition process.

A mutation within the predicted ATP-binding site (K340>H; Fig. 2) produces a mutant which has completely lost its inhibitory effect on HIV-1 replication. The predicted ATP-binding site has been shown to be essential for AAV replication (Chejanovsky & Carter, 1990).

To investigate the stage of HIV-1 replication at which the AAV-2 rep gene interferes, we first studied the effects of rep on the HIV-1 control region by the use of HIV-1 LTR (U3/R portion)-driven CAT constructs. In a first series of experiments CAT expression levels were measured in the presence of the intact AAV rep gene, but in the absence of the HIV-1 activator tat in the assay described above with 100 microinjected SW480 cells (Fig. 3). The AAV mutant pTAV2-6 (rep+), but not pTAV2-8 (rep−) inhibited HIV-1 LTR-driven CAT expression in a dose-dependent manner (Table 1). Similar results were obtained with rep constructs driven by the HCMV IE promoter/enhancer. These results

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**Table 1. Inhibition of HIV-1 U3/R-driven CAT gene expression by the rep+ AAV mutant pTAV2-6**

| Concentration of pTAV DNA (ng/μl) | Molar ratio pTAV/pHIV-CAT | Relative CAT expression
|----------------------------------|--------------------------|------------------------
| Tat absent                        |                          |                        |
| 0                                | 0.0                      | 1.0 ± 0.2              | α0.2 1.0 ± 0.2 1.0     |
| 41.5                             | 0.6                      | 0.9 ± 0.2              | 1.3 ± 0.3 1.7          |
| 83.0                             | 1.3                      | 0.8 ± 0.1              | 1.5 ± 0.2 1.9          |
| 166.0                            | 2.5                      | 0.3 ± 0.04             | 2.8 ± 0.4 9.3          |
| 332.0                            | 5.0                      | <0.05                  | 0.9 ± 0.77 >18.0       |
| Tat present (pExCMVtat, 2.5 ng/μl) |                          |                        |
| 0                                | 0.0                      | 3.7 ± 0.8              | 2.6 ± 0.3 0.7          |
| 41.5                             | 0.6                      | 3.6 ± 0.8              | 4.2 ± 0.3 1.2          |
| 83.0                             | 1.3                      | 4.3 ± 1.0              | 3.3 ± 0.1 0.8          |
| 166.0                            | 2.5                      | 5.0 ± 0.2              | 3.5 ± 0.4 0.7          |
| 332.0                            | 5.0                      | 4.9 ± 0.1              | 5.0 ± 0.6 1.0          |

* As a control the rep− AAV-2 mutant pTAV2-8 was used. SW480 cells were microinjected with a mixture consisting of pHIV-CAT (41.5 ng/μl), the rep+ (pTAV2-6) or the rep− (pTAV2-8) mutant DNA (41.5, 83.0, 166 and 332 ng/μl) and pExCMVtat (2.5 ng/μl). Bluescript DNA was added to a final total DNA concentration of 373.5 ng/μl in all experiments. Cells were harvested 24 h post-injection and CAT activities were measured in total lysates as described (Szcakiel et al., 1990). Other rep− DNAs (e.g. pTAV2-3; Fig. 3) led to the same relative CAT expression as was observed with pTAV2-8.
suggest that the U3/R portion of the HIV-1 LTR used in the HIV LTR–CAT construct contains elements involved in rep-mediated inhibition of HIV-1 gene expression. Since CAT expression driven by the HCMV IE promoter/enhancer element was not affected by the rep gene encoded by pTAV2-6, we assume that the inhibition of pHIV-CAT-mediated CAT expression does not involve any CAT sequences.

In a second series, the influence of rep on HIV-1 LTR-driven CAT expression was measured in the presence of tat expressed from the co-injected plasmid pExCMVtat. This plasmid expresses functional tat under the control of the HCMV IE promoter enhancer element from a cDNA sequence inserted into pKEX (Rittner et al., 1991). The moderate level of tat-mediated trans-activation of the HIV LTR (approx. fourfold activation) was chosen such that one could assume that even a small rep-mediated effect could be detected. Nevertheless, we did not observe any inhibitory effect of rep expressed from the AAV promoters on HIV-1 LTR-driven CAT expression in the presence of tat (Table 1). Moreover, there seemed to be a slight enhancement of CAT expression with increasing amounts of pTAV DNA which we cannot explain. In the presence of large amounts of rep expressed from the HCMV promoter-based vector (M1 DNA, 100 ng/μl; pHIV-CAT, 50 ng/μl; pExCMVtat, 2-5 ng/μl) a greater than fourfold reduction of CAT expression was seen. This might indicate that only relatively strong expression of rep can lead to down-regulation of tat-activated HIV-1 LTR-driven CAT expression. Thus it is not surprising that the relatively weak expression of rep from the AAV-2 p5 promoter (e.g. pTAV2-6) is not inhibitory. However, we cannot exclude the possibility that tat expression is also down-regulated in the presence of relatively high concentrations of construct M1.

In conclusion, the genetic analysis demonstrates that the anti-HIV-1 activity of AAV-2 can be assigned to the p78rep/p68rep expression products of a functional AAV-2 rep gene. For this inhibition (>98%), a 20-fold molar excess of cloned AAV-2 DNA was necessary, probably due to very low rep expression levels from the AAV p5 promoter. In the case of HCMV-driven rep gene expression, smaller amounts of rep expression plasmids were necessary. However, the inhibition of HIV-1 LTR-driven CAT expression which could be achieved by using cloned AAV-2 DNA as well as by using eukaryotic rep expression plasmids took place in the absence of any tat protein. This situation in microinjected SW480 cells is comparable to latent or very early stages of HIV-1 infection, with no or very low transcriptional activity of integrated proviral HIV-1 DNA. In the presence of tat, inhibition of HIV-1 LTR-driven cat gene expression by AAV rep+ DNAs was observed only with high excesses of rep over tat expression plasmids. This indicates that although basal levels of HIV-1 expression can be
inhibited successfully by AAV-2-encoded rep, elevated, i.e. tat-activated levels of HIV-1 expression, cannot be or can be only incompletely suppressed by AAV rep+ constructs. However, high level rep expression can compete against tat-activated HIV LTR-driven CAT expression, suggesting that relative intracellular levels of tat and rep might determine whether the outcome is activation or repression. This interpretation could imply that tat and rep interfere or compete with each other for the same binding site or auxiliary factors.

The inhibition of HIV-1 LTR-driven CAT expression by the rep gene shows that HIV-1 gene expression could be the target step for inhibition of the HIV-1 replication cycle. In searching for a possible target element for the rep protein(s) within the HIV-1 LTR, we identified a sequence element similar to a nucleotide sequence of the AAV-2 terminal repeat sequence (HIV-1: 5’ CTGGGAGCTCTCTGGCTAACTAGGG 3’) which is known to form an intramolecular double strand at the ends of the single-stranded AAV genome. The resulting hairpin structures have been shown to bind purified rep58 (Im & Muzyczka, 1990, 1992; Snyder et al., 1990). Interestingly, the HIV-1 TAR element forms a similar secondary structure at the RNA level. Thus, binding of rep proteins to this element could occur at the DNA or RNA level.

The molecular mechanisms underlying the strong rep-mediated inhibition of HIV-1 replication are of particular interest considering the potential role of the rep gene as an effective and possibly therapeutic agent. The AAV-2-encoded rep proteins or portions thereof are possible candidates for intracellular immunization against HIV-1 infection (Baltimore, 1988); it has been shown recently that intracellular expression of HIV-1 antisense RNA (Rhodes & James, 1990; Szczakiel et al., 1991) and trans-dominant HIV-1-encoded proteins (Malim et al., 1989; Trono et al., 1989) or RNA (Sullenger et al., 1990) inhibit HIV-1. For example, strong long-term suppression of HIV-1 replication is observed in human T lymphoid cell clones showing stable rep expression (G. Szczakiel, unpublished results).

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References


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