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**INTERACTION BETWEEN THE DNA REPAIR MACHINERY AND THE
ADENO-ASSOCIATED VIRUS (AAV):
VECTOR TRANSDUCTION AND SITE-SPECIFIC INTEGRATION**

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**This thesis is submitted for the degree of Doctor of Philosophy
in the Faculty of Life and Biomolecular Sciences of the Open University, UK**

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1.1 INDEX

| | |
|--|----|
| 1. INDEX | 2 |
| 2. ABSTRACT | 7 |
| 3. INTRODUCTION | 8 |
| 3.1. The Adeno-Associated Virus Type 2 (AAV-2) | 8 |
| 3.1.1. The Adeno-associate virus genome | 11 |
| 3.1.2. AAV-2 Rep proteins and their biochemical activities | 15 |
| 3.1.3. Adeno-associated virus 2 transcription | 17 |
| 3.1.4. Adeno-associated virus 2 replication | 20 |
| 3.1.5. Adeno-associated virus 2 integration | 23 |
| 3.1.6. Viral helper functions | 25 |
| 3.1.7. Effects of Rep proteins on transcription, cell cycle and cell transformation | 26 |
| 3.2. Genome stability – Recognition and repair of DNA Double stand breaks | 28 |
| 3.2.1. Early event after DNA DSB breaks and sensing | 29 |
| 3.2.2. DNA Double stand break repair pathways | 31 |
| 3.2.2.1. DNA repair by homologous recombination .. | 32 |
| 3.2.2.2. Non-homologous end-joining | 33 |
| 3.2.2.3. Single strand annealing | 34 |
| 3.3. Adeno-associated virus and the DNA Repair machinery | 37 |
| 4. AIM OF THE STUDY | 41 |
| 5. MATERIALS AND METHODS | 42 |
| 5.1. Cell culture and transfection | 42 |
| 5.2. Plasmids and production of stably transfected cell line | 42 |
| 5.3. Production of rAAV stocks | 44 |
| 5.4. Vector transduction | 45 |
| 5.5. Confocal microscopy | 45 |
| 5.6. Immunofluorescence | 46 |
| 5.7. RNA interference | 47 |
| 5.8. Western blotting | 48 |
| 5.9. Real-time PCR quantification of AAV site-specific integration | 49 |
| 5.10. Cell cycle profiling | 50 |
| 6. RESULTS | 51 |
| 6.1 Interaction between recombinant Adeno-associated virus (rAAV) and cellular DNA proteins: Effect on vector transduction | 51 |
| 6.1.1. Visualization of the nuclear sites of double-stranded AAV DNA formation in live cells | 51 |
| 6.1.2. Characterization of dsDNA recombinant AAV foci | 54 |
| 6.1.3. rAAV foci form in close proximity with cellular Mre11-Rad50-Nbs1 (MRN) complex repair foci | 54 |
| 6.1.4. Nbs1 inhibits the formation of dsDNA rAAV foci | 57 |
| 6.1.5. Nbs1 inhibits rAAV transduction | 59 |
| 6.1.6. Nbs1 inhibitory role on rAAV transduction requires ATM function | 59 |
| 6.1.7. Adenovirus E4orf6 and E1B55k mediated degradation of MRN complex increases rAAV transduction | 60 |
| 6.1.8. E1B55k/E4orf6 mediated degradation of MRN complex correlates with increased number of cells with dsDNA rAAV foci | 65 |

| | |
|--|-----|
| | 3 |
| 6.1.9. AAV foci form in close proximity of Mdc1 foci | 66 |
| 6.1.10. Silencing of Mdc1 decreases rAAV transduction | 66 |
| 6.1.11. Rad52 silencing results in decreased rAAV transduction efficiency | 67 |
| 6.1.12. DNA-PKcs silencing results in decreased rAAV transduction efficiency | 68 |
| 6.1.13. H2AX silencing results in decreased rAAV transduction efficiency | 73 |
| 6.2. Interaction between AAV Rep containing recombinant Adeno- associated virus (rAAV) and cellular DNA machinery: Effect on vector transduction and site-specific integration | 73 |
| 6.2.1. Establishment of a quantitative real-time PCR procedure for the determination of rAAV site-specific integration | 74 |
| 6.2.2. Silencing of Nsb1 increases frequency of Rep- dependent site-specific integration | 75 |
| 6.2.3. Silencing of Mdc1 increases frequency of Rep dependent site-specific integration | 76 |
| 6.2.4. Silencing of Rad52 increases frequency of Rep dependent site-specific integration | 81 |
| 6.2.5. Silencing of DNA-PKcs decreases frequency of Rep- dependent site-specific integration | 81 |
| 6.2.6. Silencing of H2AX decreases frequency of Rep- dependent site-specific integration | 84 |
| 6.3. Cell cycle profile after silencing of Nbs1, Mdc1, H2AX, Rad52 and DNA-PKcs | 84 |
| 7. DISCUSSION | 88 |
| 7.1. DNA Repair machinery and its influence on rAAV transduction and genome processing | 88 |
| 7.2. DNA Repair machinery and AAV site-specific integration | 95 |
| 7.3. Final considerations | 99 |
| 8. ACKNOWLEDGMENTS | 103 |
| 8. BIBLIOGRAPHY | 104 |

1.2 INDEX OF FIGURES

| | | |
|---------------------|---|-----|
| Figure 3.1. | The Adeno-associated life cycle | 10 |
| Figure 3.2. | The AAV-2 genome and its transcription | 13 |
| Figure 3.3. | The AAV-2 Inverted terminal repeat | 14 |
| Figure 3.4. | Functional domains of AAV-2 Rep | 19 |
| Figure 3.5. | Diagram of AAV Replication | 22 |
| Figure 3.6. | Diagram of homologous recombination repair | 35 |
| Figure 3.7. | Diagram of non-homologous end-joining repair | 36 |
| Figure 6.1. | Visualization of the nuclear sites of single-stranded to double-stranded AAV-Lac0.14 conversion (AAV foci) in live GFP-LacR cells | 53 |
| Figure 6.2. | Colocalization of AAV foci Nsb1 and Phospho-Serine 343 Nsb1 foci | 55 |
| Figure 6.3. | Co-localization of AAV foci Mre11 and Rad50 Nsb1 foci | 56 |
| Figure 6.4. | Silencing of Nbs1 increases rAAV foci formation | 61 |
| Figure 6.5. | Silencing of Nbs1 increases rAAV transduction | 62 |
| Figure 6.6. | AT5 cells are more permissive to rAAV and Nbs1 inhibition role requires ATM function | 63 |
| Figure 6.7. | Adenovirus E4orf6/E1b55k mediated degradation of MRN complex increases rAAV transduction | 64 |
| Figure 6.8. | E4orf6/E1B55k mediated degradation of MRN complex correlates with an increase in the number of cells with rAAV foci but not the number of foci per cell | 69 |
| Figure 6.9. | Co-localization of AAV foci with Mdc1 foci and its silencing increases rAAV transduction | 70 |
| Figure 6.10. | rAAV transduction after Rad52, DNA-PKcs and H2AX siRNA mediated silencing | 71 |
| Figure 6.11. | Establishment of real-time PCR for the quantification of AAV site specific integration: Real-time PCR quantification wide range of linearity of detection in the amplification of pAAVS1-TR | 77 |
| Figure 6.12. | Establishment of real-time PCR for the quantification of AAV site-specific integration: PCR Amplification of AAV and AAVS1 junctions only occur when Rep68 is provided | 78 |
| Figure 6.13. | Effect of Nbs1 silencing on AAV-Rep-GFP transduction and site-specific integration | 79 |
| Figure 6.14. | Effect of Mdc1 silencing on AAV-Rep-GFP transduction and site-specific integration | 80 |
| Figure 6.15. | Effect of Rad52 silencing on AAV-Rep-GFP transduction and site-specific integration | 82 |
| Figure 6.16. | Effect of DNA-PKcs silencing on AAV-Rep-GFP transduction and site-specific integration | 83 |
| Figure 6.17. | Effect of H2AX silencing on AAV-Rep-GFP transduction and site-specific integration | 86 |
| Figure 6.18. | Cell cycle profile after siRNA mediated silencing against H2AX, Mdc1, Nsb1, Rad52 and DNA-PKcs | 87 |
| Figure 7.1. | Model of rAAV interaction with cellular double strand break repair machinery | 102 |

1.3 LIST OF ABBREVIATIONS

| | |
|----------------|--|
| γ -H2AX | Phosphorylated H2AX at Serina 139 |
| 53BP1 | p53 binding protein 1 |
| aa | Amino-acids |
| AAV | Adeno-associated virus |
| AAV-2 | Adeno-associated virus serotype 2 |
| AAV-GFP | Recombinant AAV vector coding for GFP |
| AAV-LacO.14 | AAV vector containing 112 LacO repeats |
| AAV-LacZ | Recombinant AAV vector coding for β -galactosidase |
| AAV-Rep-GFP | Recombinant AAV vector coding for GFp and AAV Rep |
| AAVS1 | Adeno-associated site 1 located at 19q13.3-qter |
| ATM | Ataxia telangiectasia mutated |
| ATR | Ataxia-telangiectasia-mutated and Rad 3-related |
| ATRIP | ATR interacting protein |
| bp | Base pair |
| Cap | AAV structural Cap proteins |
| Cdk1 | Cyclin-dependent kinase 1 |
| Cdk2 | Cyclin-dependent kinase 2 |
| Ch19 | Chromosome 19 |
| Chk1 | Checkpoint 1 protein |
| Chk2 | Checkpoint 2 protein |
| DDR | DNA damage response |
| DNA | Deoxyribonucleic acid |
| DNA-PK | DNA dependent protein kinase |
| DNA-PKcs | DNA dependent protein kinase catalytic subunit |
| ds | Double-stranded |
| DSBs | Double strand breaks |
| dsDNA | Double stranded |
| E1A | Adenovirus E1A protein |
| E1b55k | Adenovirus E1b isoform 55k protein |
| E2A | Adenovirus E2A protein |
| E4orf6 | Adenovirus E4 open reading frame 6 protein |
| EGFP | Enhanced green fluorescent proteins |
| GFP | Green fluorescent protein |
| GFP-LacR | GFP Lactose repressor fusion protein |
| H2AX | Histone H2A family, member X |
| HPV | Human papilloma virus |
| HPV16 | Human papilloma virus type 16 |
| HR | Homologous recombination |
| HSV-1 | Herpes simplex virus 1 |
| HU | Hydroxyurea |
| ICP0 | Herpes simplex virus ICP0 protein |
| IR | Ionizing radiation |
| ITR | Inverted terminal repeat |
| LacO | Lactose operator |
| LacR | Lactose repressor |
| LacZ | β -galactosidase gene |
| Mdc1 | Mediator of DNA damage checkpoint 1 |
| MOI | Multiplicity of infection |
| Mre11 | Meiotic recombination 11 protein |

| | |
|------------|---|
| MRN | Mre11-Rad50-Nbs1 |
| mRNA | Messenger RNA |
| Nbs1 | Nijmegen breakage syndrome 1 (nibrin) |
| NHEJ | Non-homologous end joining |
| ONPG | o-nitrophenyl-D-galacto-pyranoside |
| ORF | Open reading frame |
| PCNA | Proliferating nuclear antigen |
| PCR | Polymerase chain reaction |
| PNK | Polynucleotide kinase |
| pSR | Plasmid Super-Retro |
| qPCR | Quantitative polymerase chain reaction |
| rAAV | Recombinant adeno-associated virus |
| Rad50 | Rad52 epistatic group protein number 50 |
| Rad52 | Rad52 epistatic group protein number 52 |
| rAdE4orf6 | Recombinant Adenovirus vector coding for E4orf6 protein |
| RBS | Rep binding site |
| RCR | Rolling circle replication |
| Rep | AAV non-structural Rep proteins |
| Rep40 | AAV Rep isophorm 40 |
| Rep52 | AAV Rep isophorm 52 |
| Rep68 | AAV Rep isophorm 68 |
| Rep78 | AAV Rep isophorm 78 |
| RFC | Replication factor C |
| RLU | Relative light units |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RPA | Replication protein A |
| shRNA | Short hairpin RNA |
| siDNA-PKcs | Short interference RNA against DNA-PKcs |
| siH2AX | Short interference RNA against H2AX |
| siLuc | Short interference RNA against Luciferase |
| siMdc1 | Short interference RNA against Mdc1 |
| siNbs1 | Short interference RNA against Nbs1 |
| siRad52 | Short interference RNA against Rad52 |
| siRNA | Short interference RNA |
| ss | Single-stranded |
| SSA | Single strand annealing |
| ssDNA | Single stranded DNA |
| TNNT1 | Slow skeletal troponin |
| trs | Terminal resolution site |
| UL29 | ssDNA binding protein, also known as ICP8 |
| vgp | Viral genome particles |
| WRN | Werner syndrome protein |

2. ABSTRACT

Adeno-associated virus type 2 (AAV-2) is a nonpathogenic, replication defective parvovirus containing a single-stranded DNA genome of 4.7 kb. Despite the increasing utilization of recombinant vectors derived from this virus (rAAV) in gene transfer applications, several aspects of the biology of both the wild type virus and of its vectors remain poorly understood. Here we developed a technique to visualize the conversion of rAAV vector genomes from single-stranded (ss) to double-stranded (ds) DNA in real time. We report that rAAV DNA accumulates into discrete foci inside the nucleus. These rAAV foci are defined in number, increase in size over time after transduction, and their presence correlates with the efficiency of cell transduction. These structures overlap with, or lie in close proximity to, the foci in which proteins of the MRN (Mre11-Rad50-Nbs1) complex and Mdc1 accumulate after DNA damage. Silencing of Nbs1 or Mdc1 by RNA interference markedly increases the formation of rAAV foci, the extent of rAAV transduction, and AAV Rep dependent site-specific integration at chromosome 19q.13.3-qter, so called AAVS1 locus. The adenovirus E4orf6 and E1B55k proteins-mediated degradation of the MRN complex also correlated with high levels of rAAV transduction and foci formation. Taken together, these observations indicate that the MRN complex plays an inhibitory role at the level of rAAV ss to double-stranded DNA (dsDNA) genome conversion, vector transduction, and site-specific integration. On the other hand, similar experiments using siRNAs against histone H2AX, Rad52, and DNA-PKcs indicated that these factors are required for effective rAAV transduction and site-specific integration.

3. INTRODUCTION

3.1. The Adeno-Associated Virus Type 2 (AAV-2).

Adeno-Associated Virus type 2 (AAV-2) was first discovered as a contaminant of Adenovirus stocks produced in African green monkey kidney cells (Hoggan et al., 1966). In fact, AAV-2 is unique among human viruses because its productive replication in cell culture is strictly dependent on the concomitant superinfection of the host cells by another non-related helper virus (Adenovirus or Herpes Simplex Virus) (Buller et al., 1981; Hoggan et al., 1966). The helper functions can also be provided by a wide variety of genotoxic agents, in which case AAV can replicate at low levels (Yakobson et al., 1987). In the absence of helper functions, AAV establishes a latent infection in the host cell by integrating site specifically on the long arm of human chromosome 19 (19q13.3-qter) (Kotin et al., 1992; Kotin et al., 1991; Kotin et al., 1990; Samulski et al., 1991). Once integrated, the provirus is passively replicated along with cellular DNA, until a new helper stimulus is provided (Cheung et al., 1980; Hoggan et al., 1966). In this case, the integrated provirus can be rescued from its chromosomal state and start replicating again (Cheung et al., 1980) (see Figure 3.1).

Given the need for viral helper functions for its fully replication activity, and since it is one of the smallest DNA viruses known so far, AAV was originally classified as a member of the *Parvoviridae* family, genus *Dependovirus* (Siegl et al., 1985). Furthermore, more recently it was possible to isolate different AAVs based on their serological properties (from AAV-1 to AAV-9, for a review see (Gao et al., 2005; Wu et al., 2006)). Nonetheless, AAV-2 is the one that has been studied more extensively, thus we will focus our attention on this serotype, except where explicitly mentioned. Another very peculiar feature of this virus is that, despite several attempts to demonstrate a possible pathogenic role for AAV, it has

been impossible to correlate infection with any known disease, despite 90% of the adult population known to have antibodies against this virus and thus been in contact with it (Blacklow et al., 1971). More than ten years ago, a couple of reports showed the presence of AAV DNA in 40% of the samples obtained from spontaneous abortions. No follow up has been provided for this indication, and no conclusion from these data can therefore be drawn (Botquin et al., 1994; Tobiasch et al., 1994).

In summary, AAV-2 cannot be considered pathogenic in humans and it can site specifically integrate at high frequency in a specific locus of the human genome.

For these reasons, and for its ability to transduce post-mitotic tissues like skeletal muscle and nervous cells, AAV-2 has emerged as one of the most promising viral vectors for gene therapy. In addition, AAV-2 is considerably less immunogenic than other viruses, such as Adenovirus, which are used in gene therapy. Thus, in recent years much of the interest in the AAV field has been devoted to the development and production of safer and more efficient rAAV vectors for the treatment of a wide array of diseases (for a recent review see (Flotte, 2004)). On the other hand, these practical applications in the Molecular Medicine field have also fuelled researchers' interest in studying AAV-2 molecular biology to get new insight into the basic mechanisms that govern its infection of the host cell.

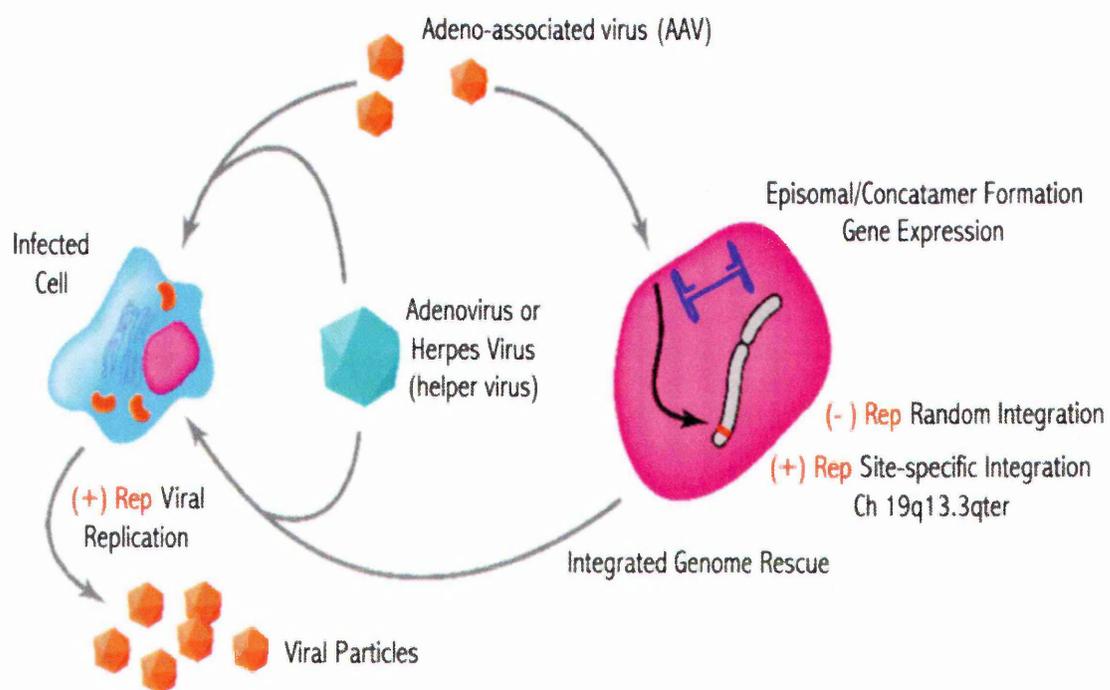


Figure 3.1. The Adeno-associated virus life cycle.

3.1.1. The Adeno-associated virus genome.

The AAV genome is a single stranded DNA molecule of 4,679 nucleotides (Srivastava et al., 1983), and contains three main genetic elements: two 145 base-pair (bp) inverted terminal repeats (ITR) that are present at each end of the genome and two open reading frames, called Rep and Cap (Lusby et al., 1980) (see Figure 3.2).

The ITRs are formed by a palindromic sequence of 125 nucleotides (A) (nucleotides 1-41 and 85 to 125) interrupted by two shorter palindromes (B and C) (nucleotides 42 to 84) (see Figure 3.3). To maximize base pairing and thermodynamic stability, each ITR can fold on itself forming a T shaped closed hairpin. Furthermore, one of the ITR in its closed, base paired conformation provides a free 3' OH for the start of DNA replication. In addition, the ITR contains three DNA sequences that are important for binding of the large Rep proteins: the Rep binding site (RBS), which is a tetrad-repeat of the GAGC sequence (Chiorini et al., 1994a; Chiorini et al., 1994b; Owens et al., 1993); the RBS', in one of the two bulges of the T hairpin (Brister and Muzyczka, 2000), and the terminal resolution site (trs) between nucleotides 124 and 125, which is site and strand specifically nicked by Rep during the process of AAV replication (Im and Muzyczka, 1990). All these features make the ITR the only *in cis* signal necessary for AAV replication, transcription, site-specific integration and rescue of the provirus from the dsDNA integrated state.

The AAV genome is transcribed from three promoters, p5, p19 and p40 that produce several polyadenylated mRNAs (Green and Roeder, 1980; Laughlin et al., 1979) (See Figure 3.2). Rep 78 and Rep68 are produced by alternative splicing of a single immature mRNA that is transcribed from the p5 promoter (Green and Roeder, 1980). The same gene is transcribed from a downstream

promoter, p19, and an alternative start site is used to produce Rep52 and Rep40 by alternative splicing. It is worth mentioning that all these four proteins are colinear and thus the two shorter isoforms represent the C-terminus of the larger ones.

The Cap ORF is transcribed from the p40 promoter and produces two alternatively spliced mRNAs, from which the three capsid proteins (VP1, VP2 and VP3) are generated, due to the presence of a non canonical ATG start site for translation in one of them (Becerra et al., 1988).

Originally, the left ORF was named Rep because several mutations in this region led to the generation of a replication incompetent AAV (Hermonat et al., 1984). Later on, these initial results could be rationalized by considering that these mutations affected the two larger Rep proteins (Rep78 and Rep68). On the contrary, viruses in which the AUG start site of the shorter Rep proteins (Rep52 and Rep40) was mutated to GGG were still proficient in replication but did not form infectious particles (Chejanovsky and Carter, 1989). This phenotype was similar to the one observed in some cap mutants, which in fact were still able to replicate but failed to accumulate ssDNA viral genomes, a process associated with packaging (Hermonat et al., 1984).

Recombinant AAV (rAAV) vectors can be easily produced by replacing all the coding regions with an expression cassette for the gene of interest flanked by the ITRs of the wild type virus (Hermonat and Muzyczka, 1984; Tratschin et al., 1984). Their natural replication incompetence, the lack of identified pathogenicity, the simplicity of their genetical manipulation, as well as the possibility to produce viral vectors that do not contain any gene from the wild-type virus, make the rAAV vectors one of the most attractive viral vectors in the gene therapy arena.

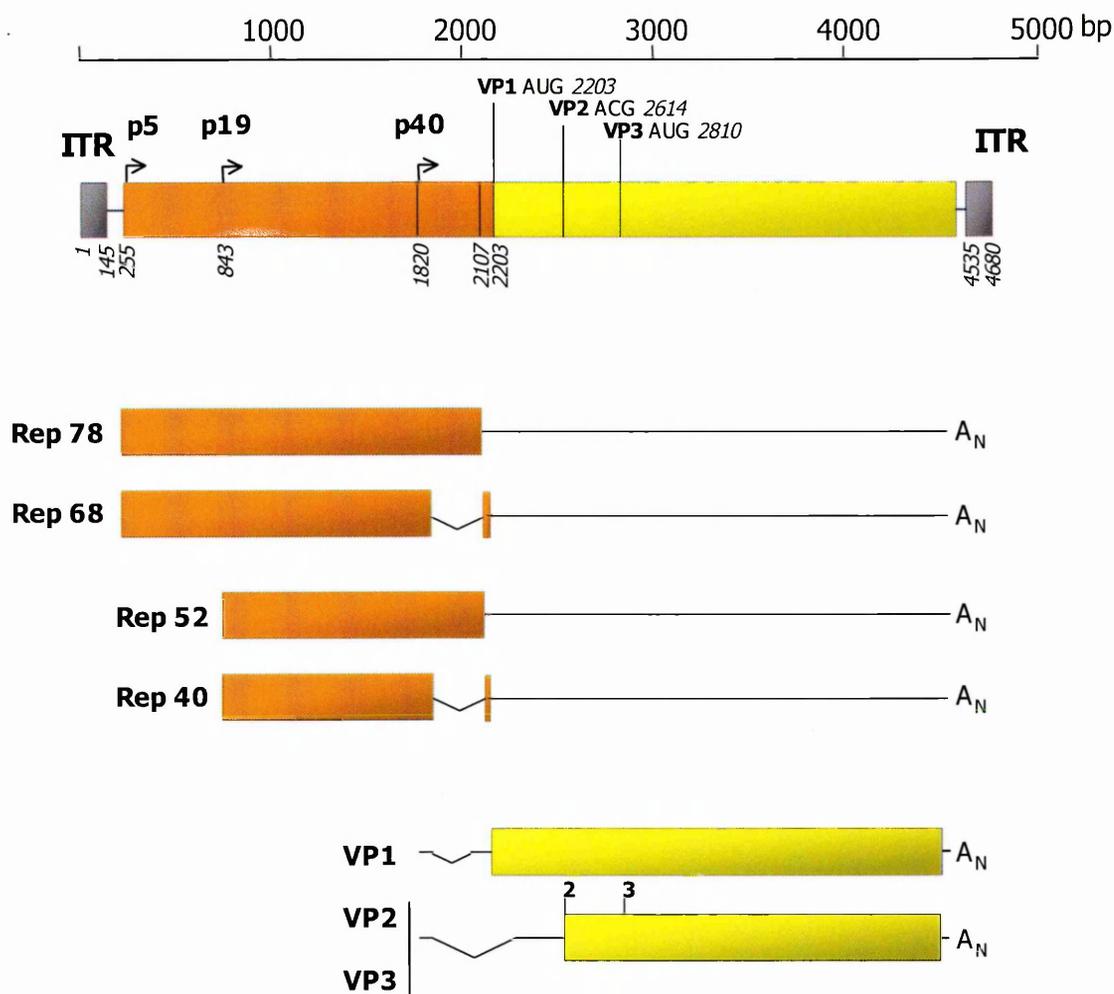


Figure 3.2. The AAV-2 genome and its transcripts.

The main genetic elements of the AAV-2 genome, together with the transcripts produced from promoters p5, p19 and p40 are indicated. The positions of the inverted terminal repeats (ITR), of the promoters and of the start sites of Cap proteins are also represented.

3.1.2. AAV-2 Rep proteins and their biochemical activities.

Rep78, Rep68, Rep52 and Rep40 were named according to their apparent molecular weight on SDS-PAGE gels. Various biochemical activities have been ascribed to the Rep proteins and now a great deal of information has been gathered on their function (Figure 3.4).

Rep68 and Rep78, which are 536 and 621 amino-acids (aa) long, are endowed with specific dsDNA binding activity toward the ITR (Ashktorab and Srivastava, 1989; Im and Muzyczka, 1989; Im and Muzyczka, 1990; Owens et al., 1993; Snyder et al., 1993), while Rep52 and Rep40 are not (Im and Muzyczka, 1992). Several residues have been shown to be fundamental for the DNA binding activity of Rep. These include Arg 107 and Arg 138, which make contacts with DNA bases, and Lys 136, which forms a salt bridge with the phosphate backbone of DNA (Hickman et al., 2004). The RBS is the minimal requirement for Rep binding to the ITR, however, in the presence of the hairpin that contains the RBS sequence, the efficiency of nicking increases up to 100 fold, (Brister and Muzyczka, 2000; Chiorini et al., 1994b; Ryan et al., 1996). Rep78 and 68 also possess site- and strand-specific nicking activity toward the trs sequence (Im and Muzyczka, 1990). This activity can be mapped to the first 208 aa of Rep78, which are sufficient when the trs sequence is in single-stranded conformation (Yoon-Robarts and Linden, 2003). In particular, Tyr156 of Rep is actively involved in the catalysis through a trans-esterification reaction. As a result of the DNA cleavage, this residue remains covalently linked to the 5' end of the cut DNA (Smith and Kotin, 2000; Snyder et al., 1990). The cutting of the trs is also dependent on metal binding, probably through two histidines (His90 and His92), Glu83 and Asp412 (Gavin et al., 1999; Hickman et al., 2002; Urabe et al., 1999; Yoon-Robarts and Linden, 2003). More recently, structural studies on the

crystallized first 197 aa of AAV-5 Rep (alone or in the presence of RBS and RBS' oligonucleotides) have provided new insight into these two biochemical activities (Hickman et al., 2002; Hickman et al., 2004). These studies have determined that the N-terminus of Rep proteins belongs to the RCR (Rolling Circle Replication) family, which includes several viral replication proteins that use a catalytic tyrosine, such as SV40 T antigen and BPV E1 (Hickman et al., 2002). These and other studies also confirmed what had been observed in the past: Rep proteins bind RBS DNA as a hexamer (Hickman et al., 2002), and the oligomerization is stimulated by ATP and DNA (Li et al., 2003; Smith et al., 1997). The region important for oligomerization can be mapped to aa 164-484, and in these 321 aa two regions are important: a putative alpha-helix bearing a 3,4-hydrophobic heptad repeat reminiscent of those found in coiled-coil domains, and the previously recognized nucleoside triphosphate-binding motif (aa 334-349) (Smith et al., 1997).

The domain comprised between aa 224-526 (the residues shared by all the four isoforms) is endowed with ATP-dependent helicase activity (Im and Muzyczka, 1990; Im and Muzyczka, 1992). Mutation K340H, which disrupts ATP binding, completely inhibits helicase activity and acts as dominant negative in AAV replication when inserted in the context of Rep78 (Chejanovsky and Carter, 1990). In contrast, the same mutation in Rep52 does indeed disrupt the ATPase activity of the protein but does not inhibit replication, probably reflecting differences in the capability of forming holocomplexes between the long and the short isoforms of Rep (Smith and Kotin, 1998). Rep K340H is also unable to inhibit transcriptional activation from the AAV p5 promoter (Kyostio et al., 1995). ATP-dependent helicase activity is also important for Rep nicking of the trs, when the latter is in a double-stranded conformation (Brister and Muzyczka,

1999; Im and Muzyczka, 1990). This last observation suggests that Rep is capable of cutting only a single stranded trs substrate, which is probably formed by DNA melting achieved through the action of the helicase domain. The two short isoforms (Rep40 and Rep52) retain ATPase and 3'-5' helicase activity (Smith and Kotin, 1998), and through these two activities they are able to encapsidate replicated AAV genomes into preformed capsids by forming a complex with the two largest Rep proteins (King et al., 2001).

The C-terminal region of Rep78 and Rep52 consists of a Zinc-Finger domain that is not present in Rep68 and Rep40. This domain is not essential for any of the Rep activities (Im and Muzyczka, 1990; Ni et al., 1994). Nevertheless, this domain is involved in the G₂/M cell cycle arrest and hyper-phosphorylation of Rb caused by the expression of Rep78 (Saudan et al., 2000). The Zinc Finger domain is also required for the interaction between Rep78 and the Serine/threonine-protein kinase X-linked (PRKX) and the inhibition of CREB-dependent transcription (Di Pasquale and Stacey, 1998).

3.1.3. Adeno-associated virus 2 transcription.

Regulation of AAV transcription has been widely studied and it appears that many layers of regulation are involved, depending on the cellular context in which AAV is present. Briefly, when the helper functions are not provided, p5, p19 and p40 are silenced. The inhibition of p5 depends on several *in cis* sequences that have been mapped; including an RBS upstream of the start site, a TATA box and a binding site for the cellular factor YY1 (McCarty et al., 1994). In the latent state, Rep78, which is produced in small amounts, actively represses p5 in an ATP-dependent manner (Kyostio et al., 1995). At the same time Rep78 can also inhibit transcriptional activity from the p19 promoter, even if there is no

strong consensus RBS sequence in this region. Thus it appears that Rep repression of promoters depends in part on its binding to the RBS, but also on its interaction with cellular transcription factors.

When Adenovirus infects the cell, the transcriptional regulation of AAV promoters is profoundly modified. By interfering with YY1 (Shi et al., 1991), Adenovirus E1A protein contributes to activate p5, which in turn produces transcripts that encode for Rep78 and Rep68 (Chang et al., 1989). In the presence of co-infecting Adenovirus and a functional p5, RBS can activate the p19 and p40 promoters, thus producing Rep52, Rep40 and the capsid proteins (Pereira et al., 1997). Rep78 and Rep68 are necessary for the Adenovirus-dependent activation of the p19 promoter, since through protein-protein interactions they promote the formation of a DNA loop that brings transcriptional activators present on p5 into the proximity of p19 (Lackner and Muzyczka, 2002); this event requires an intact p5 RBS and an intact Sp1 site upstream of p19. In the same conditions, Rep78 and Rep68 continue to repress the p5 promoter, in a sort of feedback loop (Pereira et al., 1997). The role of HSV-1 genes in the reactivation of AAV transcription has been studied less. More recent experimental evidence highlights a role for HSV-1 ICP0 in the activation of Rep transcription from a chromatin embedded p5 promoter (Geoffroy et al., 2004). Interestingly, this activity is dependent on an intact RING domain present in ICP0 (that possesses E3 ubiquitin ligase activity) and on the proteasome pathway.

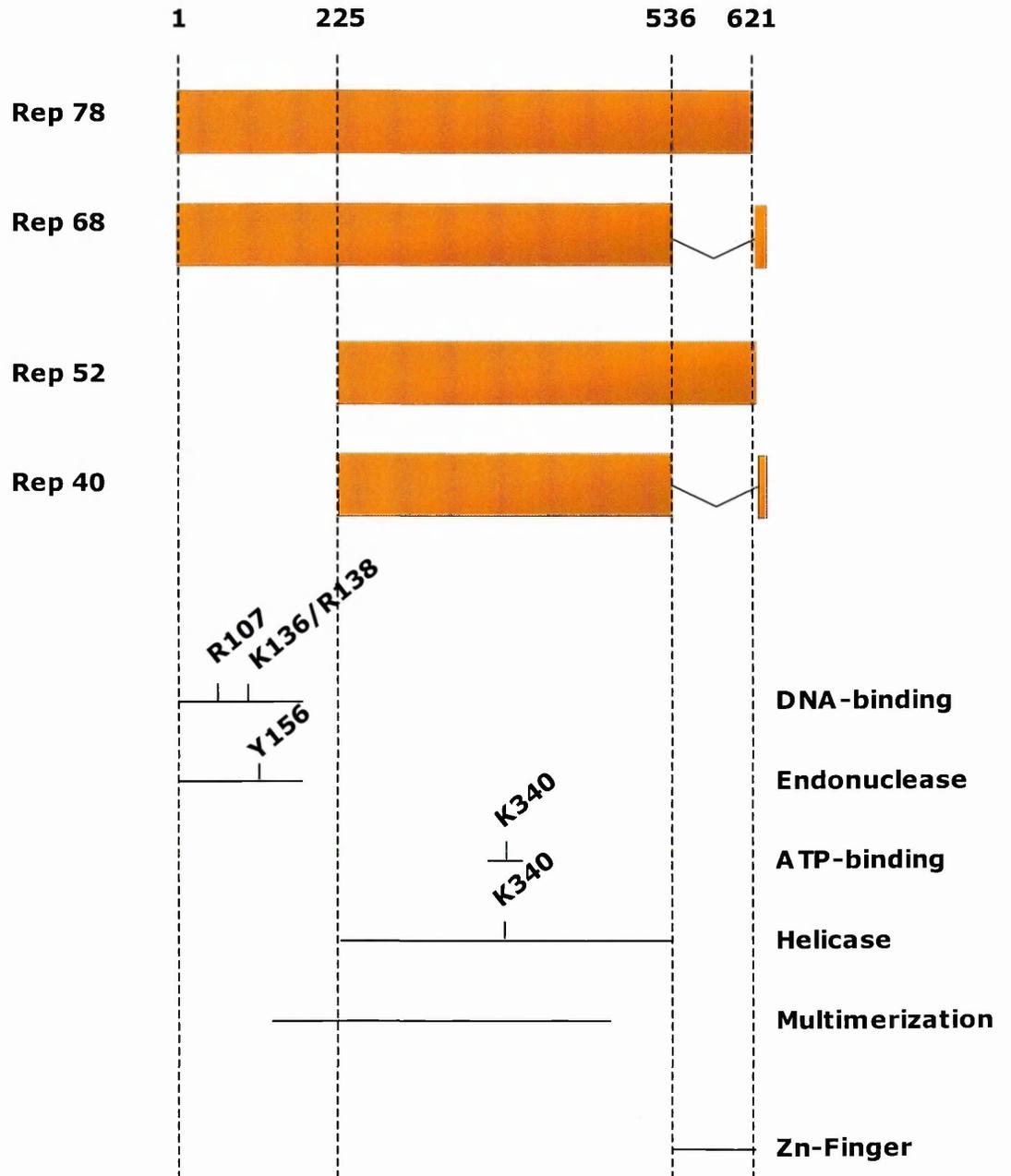


Figure. 3.4. Functional domains of AAV-2 Rep.

Schematic representation of functional domains AAV-2 Rep proteins. The position of specific residues, necessary for the various biochemical activities mentioned on the right, is indicated.

3.1.4. Adeno-associated virus 2 replication.

AAV replicates through a strand displacement mechanism that was first proposed by Tattersall and Ward (Tattersall and Ward, 1976). The AAV genome enters into the infected cell as hairpinned ssDNA. The 3' OH of one of the ITRs provides a free end for the initiation of replication of the second strand by cellular replication enzymes. The linear dsDNA molecule generated in this way is closed at one end and it must be opened to replicate all the AAV genome (Figure 3.5). This operation is carried out by Rep78 or Rep68 that bind the ITR and cut the trs at position 124 only on the original strand, thus generating a free 3' OH on the same strand and transferring the "old" hairpin onto the newly formed DNA filament. The resulting gap in the complementary strand is filled by cellular polymerases and a complete blunt ended double stranded AAV molecule is formed. At this point the newly generated ITR at the end of the original strand is displaced by the helicase activity of Rep and can fold on itself again, thus giving rise to another origin of replication. A closed dsDNA molecule is produced, resembling the intermediate generated at the first step, thus providing a substrate for subsequent rounds of replication. At the same time, a single stranded AAV genome is formed, ready to be packed into newly formed virions. Larger double stranded AAV replication intermediates corresponding to the size of dimers, trimers and tetramers are observed both *in vivo* and *in vitro* (Ni et al., 1998; Ni et al., 1994; Straus et al., 1976; Ward et al., 1998). They are the product of missing terminal resolution nicking by Rep when a new double-stranded AAV template is produced.

Several factors affect AAV replication. Either Rep78 or Rep68 must be present (Ni et al., 1994), whereas isolated Rep52 is not functional. This demonstrates that both DNA binding and trs endonuclease activities are required.

Furthermore, a cell extract of uninfected HeLa cells is not capable of supporting replication, demonstrating the need of helper functions provided by Adenovirus (Ni et al., 1994; Ward et al., 1994). In particular, it seems that these functions are not involved in increasing the efficiency of initiation of replication, but they rather augment the processivity of the reaction (Ni et al., 1994; Ward et al., 1994). Interestingly, extracts from HeLa cells that are grown at high density support replication as much as the ones obtained from Adenovirus infected cells (Ni et al., 1998). Replication is also dependent on the presence of ATP and Mg^{++} (Ni et al., 1994), a fact that mirrors the need of these factors for Rep helicase and endonuclease activity (Im and Muzyczka, 1990). The fact that cells under stress are permissive for replication strongly indicates that, beside Rep, all the factors involved in AAV replication are of cellular origin (Ni et al., 1998). The same set of studies, however, failed to reconstitute AAV replication *in vitro* entirely with purified cellular proteins. Nevertheless, it was possible to establish, by immunodepletion of nuclear extracts, that the ssDNA binding protein Replication Protein A (RPA), Proliferating nuclear antigen (PCNA), the Replication Factor C (RFC) are necessary for AAV replication (Ni et al., 1998; Ward et al., 1998). In fact, RPA binds Rep78 and Rep68 and enhance their DNA binding and endonuclease activities (Stracker et al., 2004).

So far, the only experimental approach that supported AAV *in vitro* replication only using recombinant proteins is the one described by Ward *et al.*, who used recombinant Rep and a subset of purified HSV-1 proteins (UL5, UL8, UL29, UL30, UL42, and UL52) (Ward et al., 2001).

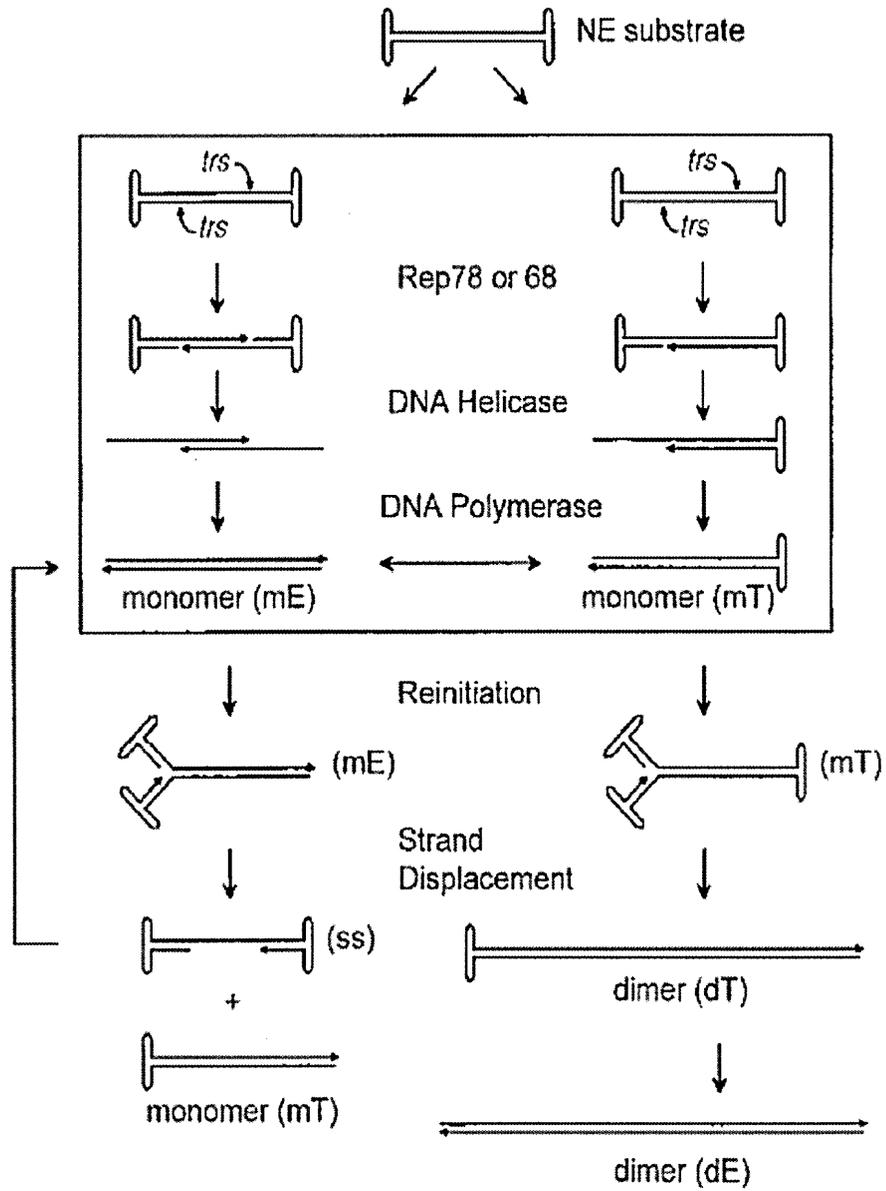


Figure 3.5. Diagram of AAV replication.

The current model established for AAV replication is shown. NE: No end substrate; trs: terminal resolution site; mE: monomer extended form; mT: monomer turnaround; ss: single strand; dE: dimer extended; dT: dimer turnaround. Adapted from (Ni et al., 1998).

3.1.5. Adeno-associated virus 2 integration.

AAV replicates and undergoes lytic infection only in the presence of a helper stimulus. Site-specific integration, on the other hand, is the mechanism that AAV employs to establish latent infection of the host cell when such helper functions are absent (Hoggan et al., 1966).

AAV is unique among human viruses because it can integrate in a specific region of the genome on chromosome 19q13.3-qter (Samulski et al., 1991). This process is strictly dependent on the presence and the activity of Rep proteins, since rAAV vectors that are devoid of viral genes integrate at random and at a lower efficiency compared to wt AAV. The region of site-specific integration in chromosome 19 was sequenced and named AAVS1 (Kotin et al., 1992). Furthermore, Dutheil *et al.* have shown that AAVS1 is in the same locus as a muscle specific gene coding for slow skeletal Troponin (TNNT1), which maps to chromosome 19.q13.4 in humans (Dutheil et al., 2000). The mouse ortholog of AAVS1 has also been identified in the mouse genome but there is still no evidence that AAV integration can occur site-specifically at this region (Dutheil et al., 2004). Sequencing of the junctions between cellular and viral DNA also showed that the integration process causes deletion, amplifications and rearrangements of viral and cellular sequences. The presence of an RBS in AAVS1 and the fact that Rep can bind both this sequence and the RBS present in the virus ITR suggests that Rep complexes might bridge together cellular and AAV sequences in the process of integration (Weitzman et al., 1994). In the same region a trs sequence is also present, and in fact Rep can nick DNA at this chromosomal site and asymmetrically replicate only one of the two strands, thus explaining the existence of extensive sequence rearrangements following AAV integration into AAVS1 (Linden et al., 1996b). Integration probably involves

some degree of replication of the chromosomal DNA, as shown by the presence of head to tail concatamers, but, surprisingly, the RBS present in the ITR of the viral genome is not necessary for this process (Young and Samulski, 2001).

The mechanisms influencing AAV site-specific integration are poorly understood. Approximately 2×10^5 RBS are present throughout the genome (Young et al., 2000). Given that Rep binds all these sites with the same affinity, the presence of RBS and trs sequences in the proper orientation and at the proper distance only in AAVS1 (and not in other regions of the human genome) might explain the target region selectivity (Young et al., 2000). Moreover, the AAVS1 region seems to be embedded in a transcriptional competent region (possibly determined by the presence of an enhancer element) and in an open chromatin conformation, thus providing accessibility to the action of Rep proteins in integration (Lamartina et al., 2000).

It is worth noting that the vast majority of rAAV vectors used in gene therapy applications do not contain the Rep ORF and thus do not integrate site-specifically

There is a growing concern that the use of viral vector in gene therapy may generate, as previously observed in clinical cases (Dave et al., 2004; Hacein-Bey-Abina et al., 2003), insertional mutagenesis in the treated patient leading to eventual cell transformation and carcinogenesis (Baum et al., 2004; Nakai et al., 2003). For this reason, understanding the mechanism of AAV Rep site-specific integration will be invaluable for the future development of new generations of viral and non-viral vectors that could target specifically their site of integration into the host genome.

3.1.6. Viral helper functions.

As mentioned earlier, AAV gene expression and productive replication are strictly dependent on helper functions provided either by other co-infecting viruses or various insults to the host cell. As far as Adenovirus is concerned, four of its proteins have been shown to provide helper functions to AAV. The product of the Adenovirus E1A gene is required for relieving Rep inhibition of the p5 and p19 promoters (Chang et al., 1989). Adenovirus VA1 RNA, instead, is probably involved in facilitating the initiation of protein translation. An Adenovirus deleted in the E2A gene (coding for the Adenovirus ssDNA binding protein) is still able to provide helper functions to AAV replication (Carter et al., 1992). Nevertheless, Adenovirus E2A seems to help AAV replication by increasing the processivity of DNA replication, by directly increasing Rep78 and Rep68 DNA binding and endonuclease activities (Stracker et al., 2004), thus possibly substituting for cellular RPA (Ward et al., 1998).

The most compelling evidences for an Adenovirus helper function have pointed toward the complex E1B55kD/E4orf6. In fact, this complex might influence the AAV replication cycle at many steps, for example by enhancing transport of mature AAV from the nucleus to the cytoplasm (Samulski and Shenk, 1988). It has been also reported that E1B55k and E4orf6 form a complex that degrades the MRN complex components and p53 protein (Stracker et al., 2002). The degradation of the MRN complex severely impairs the capacity of the cell to elicit a DNA damage response that would otherwise limit adenovirus replication (Carson et al., 2003). Furthermore, E4orf6 and E1B55k increase the transduction levels of a rAAV vector, probably by enhancing the efficiency of the second strand synthesis (Ferrari et al., 1996). Also, Weitzman and collaborators showed that Cyclin A is degraded upon E4orf6 expression, and that this correlates with an

intra S-phase arrest. This observation was correlated to rAAV transduction by demonstrating that over-expression of Cyclin A inhibited E4orf6 helper activity (Grifman et al., 1999).

As for HSV, four genes provide helper functions to AAV: the UL5/8/52 (helicase primase complex) and UL29 (ssDNA binding protein, also known as ICP8) are able to support AAV replication *in vivo* (Stracker et al., 2004; Weindler and Heilbronn, 1991) *and in vitro* (Plus UL30, the HSV polymerase) in the presence of recombinant Rep68 (Ward et al., 2001).

3.1.7. Effects of Rep proteins on transcription, cell cycle and cell transformation.

Besides playing a direct role in AAV biology, the Rep proteins are also capable of influencing the state of the host cell and to inhibit the replication of helper viruses.

First, Rep proteins induce arrest of cell proliferation that can be reversed by eliminating their expression (Yang et al., 1994). Second, it has been proven that Rep can interfere with the DNA replication of SV40 (Becerra et al., 1988), HPV16 (Marcello et al., 2000) and HSV-1 (Kleinschmidt et al., 1995). Third, Rep are able to suppress cellular transformation caused by oncogenes like c-H-ras (Batchu et al., 1994) and by oncoviruses like Adenovirus (de la Maza and Carter, 1981) and HPV16 (Human Papillomavirus16) (Walz et al., 1997). Two possible, not mutually exclusive, explanations can be invoked for these observations.

First, Rep cytostatic activity can be correlated with the ability of inducing either a S-phase (Rep78) or a G₂/M (Rep68) cell cycle arrest by increasing the levels of phosphorylated pRb and p21 (Hermanns et al., 1997; Saudan et al., 2000). Second, it is likely that Rep anti-oncogenic and anti-proliferative

properties on such different biological systems are due to the inhibitory activity of Rep on transcription. In fact, Rep does not only regulate transcription from its own promoters but it also inhibits transcriptional activity from several heterologous cellular and viral promoters such as *c-fos*, *c-myc* (Hermonat, 1994), *c-H-ras* (Batchu et al., 1994), HIV-1 LTR (Horer et al., 1995) and HPV URR (Horer et al., 1995; Marcello et al., 2000). Rep transcriptional regulation is likely achieved by different mechanisms. Promoters containing strong RBS, like AAV p5 (McCarty et al., 1994) and *c-H-ras* (Batchu et al., 1994), are directly bound by Rep and then actively inhibited by it. Nonetheless, Rep binding to DNA elements in the regulatory regions of these genes cannot be invoked as the sole mechanism to explain transcriptional inhibition. In fact some of these genes, like the HPV 18 URR and the HIV-1 LTR, do not contain an RBE in their promoter (Horer et al., 1995). In these cases it is conceivable that Rep regulates transcription through protein-protein interactions either with basal components of the transcriptional apparatus like TBP (Hermonat et al., 1998) and Sp1 (Hermonat et al., 1996), or with transcriptional co-activators like PC4 (Weger et al., 1999) and HPV16 E2 (Marcello et al., 2000).

It has been also recently reported that Rep78 inhibits Cdc25A activity by a novel mechanism, in which binding between the two proteins stabilizes Cdc25A, thus increasing its abundance, while at the same time preventing access to its substrates cyclin-dependent kinase (Cdk) 2 and Cdk1. This effect alone does not induce a complete S phase block. In addition, Rep78, as well as Rep68, produces nicks in the cellular chromatin, inducing a DNA damage response mediated by ataxia telangiectasia mutated (ATM) leading to G1 and G2 cell cycle arrest (Berthet et al., 2005).

3.2. Genome stability – Recognition and repair of DNA Double strand breaks

In eukaryotic cells, efficient surveillance mechanisms have evolved to rapidly recognize DNA damage and to signal its presence. DNA damage can dramatically impair cell function, leading to cellular transformation and carcinogenesis, or inducing cycle arrest and eventually cell death (van Gent et al., 2001). Of the various forms of damage that can be inflicted, probably the most dangerous is represented by DNA double-strand breaks (DSBs). These are created when a DNA lesion is composed of two or more strand breaks at approximately the same nucleotide pair simultaneously cuts a DNA molecule into smaller fragments. (Jackson, 2002; Khanna and Jackson, 2001; Rich et al., 2000). DSBs can be generated after cell exposure to endogenous or exogenous agents. Some of the exogenous agents that can produce DSBs are: ionizing radiation (IR), genotoxic, and radiomimetic or chemotherapeutics drugs like hydroxyurea, camptothecin, etoposide, and bleomycin, (Povirk, 1996). Among the principal endogenous agents that can lead to the formation of DSBs are the Reactive Oxygen Species (ROS), typically produced during normal metabolic reactions (Barzilai and Yamamoto, 2004; Storz, 2005). Double strand breaks can also be produced during recombination processes like meiotic recombination and, V(D)J and immunoglobulin isotype class switch recombination (Khanna and Jackson, 2001), as well as during cellular DNA replication through damaged or nicked DNA (Robison et al., 2004) or during aberrant processing of telomeres (Reaper et al., 2004).

3.2.1. Early events after DNA DSB breaks and sensing.

Detection of DNA double-strand breaks involves sensor proteins that become activated and trigger several signaling cascades. This signaling leads to the recruitment of specialized proteins to the site of damage that contribute to the subsequent repair of the damaged DNA. One of the first described events after DNA DSBs is the phosphorylation of the histone H2AX (γ -H2AX). The phosphorylation of H2AX on serine 139 occurs very rapidly and depends on ATM, ATR and DNA-dependent protein kinase (DNA-PK) (Burma et al., 2001; Mukherjee et al., 2006). γ -H2AX foci are typically visible by immunofluorescence few minutes after induction of DSBs upon treatment of cells with ionizing radiation, but they are also formed at sites of stalled replication forks (Furuta et al., 2003; Rogakou et al., 1998).

In particular, DNA double-strand breaks in chromosomal DNA elicit a signaling response through the ataxia-telangiectasia mutated (ATM) protein kinase, which coordinates cell-cycle arrest, DNA repair and apoptosis (reviewed in Shiloh, 2003; Stucki and Jackson, 2004).

Mdc1, 53BP1 and the MRN complex, constituted by the Mre11, Rad50, and Nbs1 proteins localize at nuclear foci in response to double-strand breaks. MRN complex have been implicated in DNA end-processing during homologous recombination (Trujillo et al., 1998) and possibly, but still controversially, during non-homologous end-joining (Di Virgilio and Gautier, 2005; Yang et al., 2006). *In vitro* as well as *in vivo* experiments suggest that the MRN complex may function as a bridge between double-strand break ends through interactions between the coiled-coil domains of RAD50 (Aten et al., 2004; de Jager et al., 2001; Hopfner et al., 2002).

Recent evidence indicates the MRN complex is an essential mediator of ATM recruitment to and activation by DSBs, both by forming multiple protein-protein contacts with ATM (Falck et al., 2005; Lee and Paull, 2005) and by tethering damaged DNA, thereby increasing its local concentration (Dupre et al., 2006). The retention of MRN at the DSB sites requires direct binding of its Nbs1 component to the Mediator of DNA damage checkpoint protein 1 (Mdc1) (Lee and Paull, 2005; Lukas et al., 2004). Thus, both MRN complex and Mdc1 are crucial for the efficient activation of the intra-S phase checkpoint through the activation of ATM (Lukas et al., 2004; Stucki and Jackson, 2004). Mdc1 proteins is known to increase the retention of Nbs1 and 53BP1 to the sites of damage (Bekker-Jensen et al., 2005; Lukas et al., 2004). It is also known that Mdc1 binds γ H2AX at the site of DSBs and that the two proteins appear to function in a common pathway of regulation of the cellular response to DNA DSBs (Lee et al., 2005; Stucki et al., 2005).

ATM- and Rad3-related (ATR) kinase activity is stimulated upon binding of the ATR-ATRIP complex to an RPA-ssDNA complex, thus making single-stranded DNA its primary DNA damage lesion. ATR can subsequently phosphorylate and activate the checkpoint kinase Chk1, allowing further amplification of the checkpoint signal. The ATR and Chk1 kinases then modify a variety of factors that can lead to stabilization of stalled DNA replication forks, inhibition of origin firing, inhibition of cell cycle progression, mobilization of DNA repair factors, and induction of apoptosis. It is believed that ATR is essential for cell survival due to its role in surveillance of DNA replication (Dupre et al., 2006; Zou and Elledge, 2003).

ATM and ATR are known to regulate parallel damage response signaling pathways. While ATM is normally activated by DNA double strand breaks, ATR

is thought to be recruited to regions of single-stranded regions and its activation has been associated to the stalled replications forks (Zou and Elledge, 2003). Even though the two signaling pathways have been considered to function independently, recent studies have demonstrated that ATM functions upstream of ATR following exposure to ionizing radiation (IR) in S/G2 (Jazayeri et al., 2006; Myers and Cortez, 2006; Stiff et al., 2006).

In response to DNA damage, several components of the DNA damage response, which are normally found diffuse through the nucleus, are rapidly relocalized and concentrated into nuclear complexes that are microscopically detected as foci (Seno and Dynlacht, 2004; van den Bosch et al., 2003). Although the exact nature of the molecular processes occurring at these foci is still not completely understood, experimental evidence indicates that at least some of these foci form in correspondence or in close proximity to the sites of actual DNA damage and that the increase of their local concentration is beneficial for genome surveillance and repair (Raderschall et al., 1999; Tashiro et al., 2000). It is worth noting that, among the diverse modes of protein redistribution after DSB formation, only the proteins assembled in the DSB-flanking chromatin region on the ssDNA compartments has been readily detected to form intranuclear foci, thus foci formation cannot be used as the only criteria for the direct involvement of a given protein in the DSB response (Bekker-Jensen et al., 2006).

3.2.2. DNA Double stand break repair pathways

The eukaryotic cell has evolved two main mechanisms for the repair of DSBs: the potentially error-free homologous recombination (HR) pathway and the error-prone non-homologous end-joining (NHEJ) pathway (Jeggo and Lobrich,

2006; O'Driscoll and Jeggo, 2006). A third pathway called single-strand annealing (SSA) is considered to be a variant of HR (van den Bosch et al., 2002). The choice of which pathway should be activated may depend on whether the damaged DNA region has already been replicated and on the precise nature of the break. NHEJ functions at all stages of the cell cycle, but plays the predominant role in both the G1- and in S-phase in regions of DNA that have not yet been replicated. HR functions primarily in repairing both one-sided DSBs that arise at DNA replication forks and two-sided DSBs arising in S or G2-phase in chromatid regions that have been replicated (O'Driscoll and Jeggo, 2006; Rothkamm et al., 2003)

3.2.2.1. DNA repair by homologous recombination

Homologous recombination (HR) repair acts on DSBs occurring within replicated DNA (replication-independent DSBs) or on DSBs that are generated at broken replication forks (replication-dependent DSBs). This pathway involves processing of the ends of the DNA double-strand break, homologous DNA pairing, strand exchange, repair DNA synthesis, and resolution of the heteroduplex molecules. A large number of proteins are known to be involved in this process; among them there are, Rad51B, Rad51C, Rad51D, Rad52, Rad54B, Rad54L, XRCC2, XRCC3, BRCA1, and BRCA2 (Symington, 2002).

One of the first events during homologous recombination repair is the resection of the DNA around the break point. This process generates tracks of ssDNA overhangs. It is believed that Mre11 nuclease activity is required in this process; however, there is not a general agreement about this point, since *in vitro* studies have shown that the Mre11 protein possesses a 3' to 5' nuclease activity,

while the resection would require nuclease activity working in the opposite direction (from 5' to 3'). These findings, however, do not rule out the possibility that Mre11 may show a different enzymatic activity *in vivo* or that the MRN complex may recruit other nucleases with a suitable activity (Wyman et al., 2004). Rad51 protein binds to the DNA overhangs, probably after displacing RPA proteins. This initial binding is followed by the recruitment of other proteins such as Rad52 and Rad54 and by searching for homologous sequences to be used as template for the repair of the damaged region. This process is followed by invasion of the damaged strand into the homologous sequence. The 3' end then works as primer for DNA synthesis that makes a copy of the template. The last step requires the resolution of a so called, Holliday junction, with or without a crossover (Sung and Klein, 2006; Symington, 2002) (see Figure 3.6).

3.2.2.2. Non-homologous end-joining.

The NHEJ pathway is initiated in response to the formation of a DNA double-strand break (DSB) induced by a DNA-damaging agent such as ionizing radiation. First, the Ku70/80 heterodimer binds the ends of the DSB. The catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) is then recruited to DNA-bound Ku protein to form the DNA-PK holoenzyme. The ends of the break are brought together as soon as two molecules of DNA-PK (one at each end of the break) are joined in a synaptic complex. Other factors, such as polynucleotide kinase (PNK), Artemis, the MRN complex, hTdp1 or the Werner Syndrome protein (WRN) may be required for processing the DNA ends prior to end rejoining (Collis et al., 2005; Jeggo and Lobrich, 2005); however, the precise timing of these events is not known. Following the formation of the synaptic

complex, the XLF/Cernunnos (Ahnesorg et al., 2006; Revy et al., 2006), XRCC4, DNA ligase IV proteins are recruited. Prior to end rejoining, protein factors must be removed from the DNA. This may involve DNA-PK autophosphorylation. At the final stage, the DNA ends are ligated and the DNA is repaired. (Cahill et al., 2006; Sekiguchi and Ferguson, 2006) (see Figure 3.7).

3.2.2.3. Single strand annealing.

Single strand annealing (SSA) is a process that is initiated when a double strand break is made between two repeated sequences oriented in the same direction. It requires a first step of 5' to 3' resection of both the DSB ends to generate long single strand overhangs, so that the complementary strands can anneal to each other. MRN complex is believed to be involved in this process of extensive resection. SSA is typically defined as a Rad51 independent, Rad52 dependent variant of HR.

A question that is often raised is why would organisms evolve a repair mechanism that deletes genetic material that might be essential for survival?. One possible explanation is that SSA is well suited to repair DSBs that occur within tandem arrays of sequences. A DSB would initiate SSA resulting in a deletion event of redundant genes. Another consideration is that SSA is only one out of several mechanisms that can seal a DSB, and offers a chance to a cell to survive if it can not repair a DNA damage using other repair pathways (Harrison and Haber, 2006).

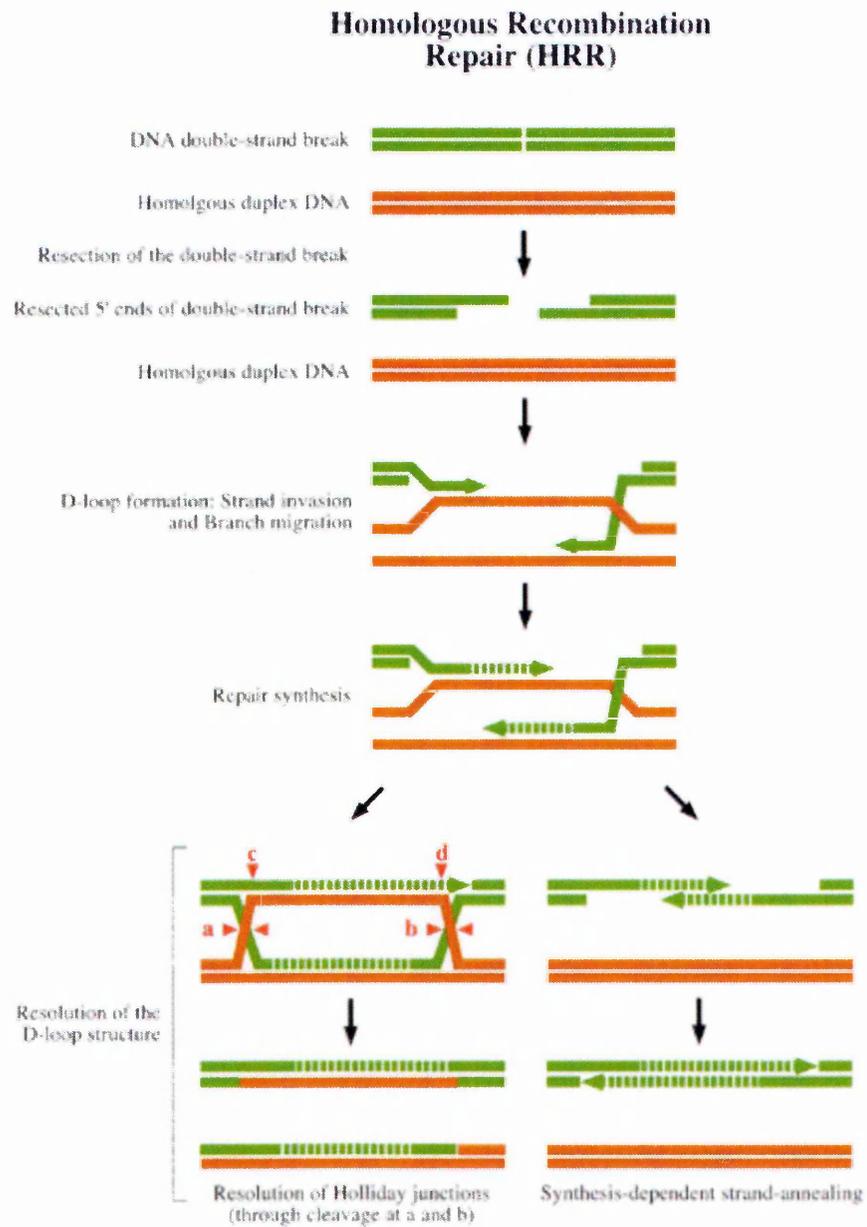


Figure 3.6. Diagram of Homologous Recombination Repair

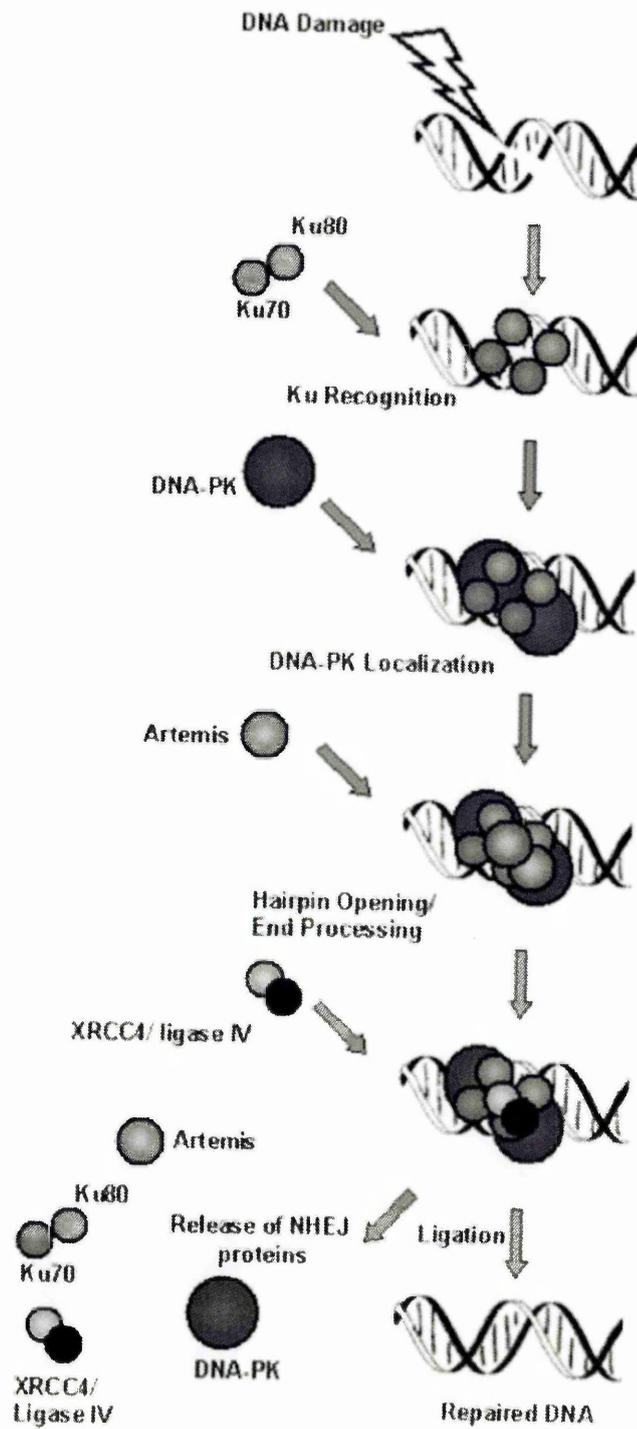


Figure 3.7. Diagram of Non-homologous end-joining repair
Adapted from Collis, *et al*, (2005)

3.3. Adeno-associated virus and the DNA Repair machinery.

Despite the growing utilization of rAAV vectors for pre-clinical and clinical gene transfer applications, several aspects of the life cycle of both wild type virus and, most notably, of the recombinant vectors still remain largely obscure. In particular, little information is available about the processing of recombinant AAV DNA once the viral particles have been internalized. This is a topic of particular relevance considering that a relatively small number of tissues are permissive to rAAV transduction, despite the receptors for AAV internalization are widespread in most cell types *in vivo* (Ding et al., 2005; Perabo et al., 2006; Wu et al., 2006).

In cultured cells, a marked increase in rAAV transduction efficiency is obtained by treating cells with agents that affect genomic DNA integrity or metabolism, such as UV irradiation, hydroxyurea (HU), topoisomerase inhibitors and several chemical carcinogens (Alexander et al., 1994b; Russell et al., 1995; Yang et al., 1994; Zentilin et al., 2001; Zhou et al., 1999). It is believed that, a major effect of these treatments is the improved conversion of the input vector ssDNA genome into dsDNA (Ferrari et al., 1996; Fisher et al., 1996), an essential requisite for gene expression.

It has been reported that cells harboring mutations in genes that participate in DNA repair, modify wt and recombinant AAV permissivity and the way its genomes are processed intracellularly. In mouse smooth muscle cells defective for DNA-PKcs rAAV genomes do not form circular episomes, and remain mainly as linear monomers or form only linear concatamers (Song et al., 2001); however, in this study, the authors did not show a significant change in the total levels of gene expression when infecting mice harboring the mutation. *In vitro* studies

have suggested a possible inhibitory role of DNA-PKcs on Rep dependent site-specific integration (Song et al., 2004).

The single-stranded nature of DNA of the AAV genomes, the presence of encapsidated genomes of either positive and negative polarity, and the secondary structure of the ITRs, make us believe that like other viruses (Sinclair et al., 2006; Weitzman et al., 2004), AAV genomes may be recognized by the cell as aberrant forms of DNA. Indeed, AAV genomes elicit a DNA damage response that does not depend on the expression of any viral protein (Raj et al., 2001) and resembles the response to an aberrant cellular DNA replication fork (Jurvansuu et al., 2005).

Human cells mutated for ATM are dramatically more permissive to rAAV transduction (Sanlioglu et al., 2000; Zentilin et al., 2001), suggesting that the DNA damage response, or at least part of it, may exert a negative effect on viral transduction. Another very interesting observation is that adenovirus proteins E4orf6 and E1B55k, which are known to increase AAV replication and rAAV transduction, specifically degrade p53 protein and the MRN complex (Stracker et al., 2002), events that dramatically impair the capacity of the cell to activate ATM and to mount an effective response after DSBs induction by ionizing radiations (Carson et al., 2003). Herpes simplex virus 1 (HSV-1), another well recognized AAV helper virus has been reported to deregulate the DNA damage signal transduction pathway (Hadjipanayis and DeLuca, 2005; Lilley et al., 2005; Wilkinson and Weller, 2006).

AAV Rep proteins are also known to interact with several cellular proteins, possibly affecting how the cell reacts to the presence of the AAV genomes. One example of this interaction is the reported direct binding between Rep78 and Cdc25A. This interaction presumably increases Cdc25A abundance

and prevents access to its substrates cyclin-dependent kinase (Cdk)1 and Cdk2 (Berthet et al., 2005). It has also been reported that Rep68 and 78 expression induces DSBs that trigger the formation of γ -H2AX foci (Berthet et al., 2005). This observation has raised the possibility that permissivity to rAAV transduction might be related to the induction of cellular DNA damage response (DDR) mechanisms. Consistent with this notion, a few years ago we observed that, after transduction, rAAV genomes physically interact with some proteins involved in the DNA double strand break (DSB) repair, in particular Rad52 and Ku86 (Zentilin et al., 2001). The presence of Rad52 protein in the rAAV genomes correlated with increased vector transduction efficiency. Recent reports have clearly demonstrated the participation of the homologous recombination machinery during rAAV mediated gene targeting, a process that may be mechanistically similar to AAV site-specific integration (Vasileva and Jessberger, 2005; Vasileva et al., 2006).

Recent work exploiting self-complementary AAV vectors (which bypass the single-stranded to dsDNA conversion step) has shown that ATM and MRN proteins are required for genome circularization (Choi et al., 2006). Thus, it might be envisioned that some cellular proteins may exert different roles after AAV genomes are converted to dsDNA. At this stage, resolution of the secondary structures in the ITRs by promoting circulation or multimerization might be essential to allow stable maintenance of the viral genomes inside the nucleus.

The finding that the cellular DDR machinery is involved in rAAV genome processing is equally compatible with the possibilities that some of the DDR proteins might exert either a positive or a negative role on single-stranded to dsDNA conversion and genome processing. If cells are treated with DNA damaging agents, it is possible that factors, that may be positive or negative for

AAV genome processing, transduction and site-specific integration are diverted from the incoming viral genomes to repair the cellular DNA.

4. AIMS OF THE STUDY

Various experimental evidence indicate that the cellular machinery controlling the DNA damage response to double strand breaks has an important impact in the life cycle of recombinant and wild-type Adeno-associated virus.

The first aim of this study was to determine the role of the cellular factors involved in the DSB damage response in the process of conversion of the recombinant AAV genomes from single-stranded to double stranded DNA, a very well known limiting step for an efficient vector transduction. For this purpose, we developed a microscopic visualization technique that allowed us to observe and characterize the nuclear sites of conversion and/or accumulation of rAAV double-stranded genomes. We used this methodology to evaluate the possible association of cellular factors that form repair foci upon DNA damage induction with the nuclear foci where dsDNA rAAV genomes accumulate

The second aim of this study was to characterize the role of the cellular proteins observed to colocalize with the rAAV foci, as well as of other proteins that participate in the homologous recombination and non-homologous end-joining repair pathways, in the process of efficient gene expression after transduction. The characterization of the cellular factors influencing this process could help us to understand the mechanism of cells permissivity to rAAV vectors.

The last but not the least aim of this study was to identify the cellular factors involved in the DNA damage response that may influence the process of AAV Rep dependent site-specific integration into a specific region of human chromosome 19.q.13.3qter, called AAVS1. Understanding this process is of great relevance for the future development of viral gene therapy vectors with genome targeting capacity, since this property could reduce the potential risk of insertional mutagenesis associated with gene transfer.

5. MATERIALS AND METHODS

5.1. Cell culture and transfection.

HeLa, MRC5 and AT5, HeLa pSuper-Retro-Mdcl and HeLa pSuper-Retro-LacZ cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal bovine serum. HeLa GFP-LacR and MRC5 GFP-LacR stably transfected cell lines were cultured as mentioned above in the presence of 300 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ of hygromycin. HeLa GFP-LacR/E1B55k and HeLa GFP-LacR/H354 cells clones were cultured in the presence of 1 $\mu\text{g/mL}$ of puromycin.

Cell were transfected with siRNAs using Gene Silencer reagent (Genlantis, San Diego, CA, USA) according to the manufacturer's procedure. DNA was transfected using Effectene transfection reagent (QIAGEN) following manufacturer's protocol.

5.2. Plasmids and production of stably transfected cell line.

To produce the pAAV-Lac0.14 vector the following procedure was followed: the plasmid p3'ssdEGFP, which expresses GFP linked to the Lac repressor (LacR) and containing a nuclear localization signal, and pSV2dhfr 8.32 which contains 10 kb of 292-bp lac operator repeat (LacO) were kindly provided by A.S. Belmont (University of Illinois at Urbana-Champaign). The plasmid pCMV-MCS (Stratagene, La Jolla, CA, USA) was digested with Not I to excise the multiple cloning site and substitute it with a multiple cloning site containing Not I, Sal I, Eco RI, Xho I, Bam HI, and Not I to obtain plasmid pMCS3'. The 292 LacO repeat, obtained digesting pSV2dhfr 8.32 with Eco RI, was cloned into the Eco RI site of pMCS3'; the 8-mer repeat was then amplified by directional

cloning (Robinett et al., 1996) to obtain a vector containing 14 LacO repeats, for a total of 112 LacR binding sites. The 14 LacO repeats were cloned into the Not I site of pAAV-MCS to generate pAAV-LacO.14. To obtain stable clones expressing the LacR fused to GFP, HeLa and MRC5 cells were transfected with polyfect transfection reagent (Qiagen GmbH, Hilden, Germany) and clones were selected with hygromycin (300 µg/ml for HeLa cells; 100 µg/ml for MRC5 cells).

pRep-GFP plasmid was constructed as follows: AAV wild type (wt) Rep gene was amplified by PCR from the wtAAV molecular clone pSub201 plasmid (kindly provided by J. Samulski, Chapel Hill, North Carolina, USA) (Samulski et al., 1987). Forward primer contained the original found restriction enzyme site for Xba I cleavage, reverse primer was synthesized to introduce a new Sma I and Xba I sites, required for the following step of cloning. The primer sequences are the following: forward primer R201F 5'-CTAGACCCGGGGTCTTATTCCTTCACAGAGAGTGTCC-3' and reverse primer R201R 5'-TCGAGGACACTCTCTGTGAAGGAATAAGACCCCGGGT-3'. PCR amplified fragment containing the Rep genes was purified and cut with Xba I restriction enzyme and cloned in a pUG7 cloning vector previously digested with the same restriction enzyme. DNA sequencing of the insert was conducted to verify its fidelity in comparison to the pSub201 sequence provided by J. Samulski. pUG7 containing wtAAV Rep gene was digested with Sma I restriction enzyme and blunted (using T4 DNA polymerase) Ase I and Afl II excised DNA fragment from pEGFP-N1 was inserted (Stratagene, Accession number U55762). The excised fragment contained the Cytomegalovirus (CMV) promoter, the Enhanced Green Fluorescent proteins (EGFP) and the SV40 polyadenylation site. Orientation of the EGFP expression cassette was verified by digestion with Xba I and Nde I restriction enzymes. pUG7 vector containing wt AAV Rep and EGFP expression

cassette was digested with Xba I restriction enzyme, the 3.7 kbp fragment containing Rep and EGFP genes was cloned in place of the Rep-Cap sequence (flanked by Xba I sites) of pSub201. In this way we generated a plasmid containing the sequence of the inverted terminal repeats (ITRs) of wt AAV and a wt Rep and EGFP expression cassette. We named this plasmid pRep-GFP.

GFP-LacR/E1B55k and GFP-LacR/H354 HeLa derived cells lines were kindly provided by M.D. Weitzman (San Diego, California, USA).

5.3. Production of rAAV stocks.

The rAAV vectors used in this study were produced from pTR-UF5 (AAV-GFP), kindly provided by N. Muzyczka (University of Florida, Gainesville, FL), pAAV-LacZ (Stratagene), pRep-GFP, and pAAV-LacO.14. Cloning and propagation of AAV plasmids was carried in XL-10 Gold E. coli strain (Stratagene, La Jolla, CA, USA). Infectious AAV vector particles were generated in 293 cells, using a dual plasmid cotransfection procedure with pDG as packaging helper plasmid (kindly provided by J.A. Kleinschmidt, Heidelberg, Germany) and the viral vector plasmid as previously described (Zentilin et al., 2001). Titration of AAV-GFP and AAV-LacZ viral particles was performed by real time PCR quantification of the number of viral genomes, as described (Zentilin et al., 2001); the viral preparations used in this work had titers between 1×10^{11} and 1×10^{12} viral genome particles (vgp) per ml. The titers of AAV-LacO.14 were measured by Southern blotting using serial dilutions of the input pMCS3'lacO.14 plasmid as a standard; the preparations of this vector had titers between 1×10^8 and 1×10^9 viral genome particles (vgp) per ml.

5.4. Vector transduction.

Transduction with AAV-GFP was performed by plating 4×10^5 cells in 24-well plates. In the experiments with AAV-LacO.14, 2×10^4 cells were seeded 24 h before vector addition in 8-well chamber slides (Labtech International, Woodside, UK) or in 6-cm glass bottom slides. Transduction with AAV-LacZ was performed in 96-well plates with 3×10^3 cells per well. Cell treatment with HU, when indicated, was performed by incubating cells for 12-16 h with 1 mM HU (Sigma-Aldrich, St. Louis, MO, USA) before the addition of vector. Transduction with the different vectors was performed in DMEM additioned with 10% FCS. After 3 h incubation, cells were washed in PBS and fresh culture medium was added. Cellular GFP fluorescence was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) 36 h after AAV-GFP addition. In the case of AAV-LacZ transduction β -galactosidase activity was determined 36 h post-infection by measuring o-nitrophenyl-D-galacto-pyranoside (ONPG) cleavage using a photometric assay, as described elsewhere (Brown et al., 2000). Relative light units (RLU) were expressed as the ratio between ONPG absorbance at 405 nm divided by the amount of proteins used in the assay (in ng/ml), multiplied by an arbitrary factor of 1,000. Protein content was determined using a BCA assay kit (Pierce Chemical. Montlucon, France)

5.5. Confocal microscopy.

Live or fixed cultures were analyzed by confocal microscopy using a Leica TCS-SL or Zeiss LSM 510 laser scanning microscopes. The different channels were detected sequentially, and the laser power and detection windows were adjusted for each channel to exclude overlap between different

fluorochromes. rAAV foci location was monitored through a z-series of images. For live cell recording, cells plated on 6-cm glass bottom dishes were placed in a humidified Plexiglas chamber and maintained at 37°C and 5% CO₂ throughout the experiment.

5.6. Immunofluorescence.

At the indicated time points post infection, cells were washed twice with PBS and fixed and permeabilized either with 2% paraformaldehyde in PBS for 15 min at room temperature followed by two washes with 0.1% Triton X-100 in PBS or with 100% methanol at -20°C for 20 min followed by treatment with 100% acetone at -20°C for 20 seconds. After fixing, the cells were washed twice, and incubated with primary antibody for 1 h and 30 min in PBS plus 0.15% glycine and 0.5% BSA (PBS⁺) in a moist chamber at room temperature. The following antibodies were used: mouse anti-human Rad50 (GeneTex, San Antonio, TX, USA, ab89) diluted 1:200; mouse anti-human Mre11 (GeneTex, ab214, 12D7) and rabbit anti-human Nbs1 (Novus Biologicals, Littleton, CO, USA, ab398, NB 100-143), rabbit anti-Phospho-Nbs1 (Ser343) (Novus Biologicals, 100-284A3) diluted 1:500 and, anti-Mdc1 rabbit polyclonal serum (kindly provided by S.P. Jackson) a diluted 1:200. Cells were incubated with the secondary antibodies for 1 h in PBS⁺ in a moist chamber at room temperature. The secondary antibodies used were goat anti-rabbit Alexa 594-conjugated and goat anti-mouse Alexa 594-conjugated (Molecular Probes, Eugene, OR, USA) both diluted 1:1000. Chamber slides were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) containing 4'-6'-diamino-2-phenylidole (DAPI). Fixed

cells were analyzed by fluorescence microscope with a Leica DMLB connected to Leica DC camera or by confocal microscopy.

5.7. RNA interference.

Cells (4×10^5) were plated in 35 cm dishes and transfected using GeneSilencer (Gene Therapy Systems, San Diego, CA, USA) according to the manufacturer's recommendations. The sequence of the siRNAs against H2AX, Mdc1, Nsb1, Rad52, DNA-PKcs and Luciferase are indicated in table MM1. All siRNA duplexes were ordered from Dharmacon (Dharmacon, Lafayette, CO, USA).

Twenty-four hours after siRNA transfection, cells were trypsinized and replated in 24-well plates, 96-well plates or 8 well-chambered slides and transduced with rAAV.

The pSR-Mdc1 cell line is a HeLa cell derivative transduced with the pSUPER-retro retroviral vector (pSR) (Brummelkamp et al., 2002) stably expressing an anti-Mdc1 shRNA (insert sequence: GATCCCCGTCTCC CAGAAGACAGTGATTCAAGAGATCACTGTCTTCTGGGAGACTTTTTGGA AAA). The control pSR-LacZ cell line expresses an anti- β -galactosidase shRNA (insert sequence: AGCTTTTCCAAAAAGTCTCCCAGAAGACAGTGATCTCT TGAATCACTGTCTTCTGGGAGACGGG) from the same vector. Both cell lines were kindly provided by Y. Shiloh (Tel Aviv, Israel)

Table 5.1. List of synthetic siRNAs

| Targeted gene | Sequence | Reference |
|--------------------|---------------------------|------------------------|
| H2AX | CAACAAGAAGACGCGAAUC(dTdT) | (Lukas et al., 2004) |
| Mdc1 | CAACAAGAAGACGCGAAUC(dTdT) | (Stewart et al., 2003) |
| Nbs1 | GGAAGAAACGUGAACUCAA(dTdT) | Provided by J. Falck* |
| Rad52 | AGACUACCUGAGAUCACUA(dTdT) | (Lau et al., 2004) |
| DNA-PKcs | GAUCGCACCUUACUCUGUU(dTdT) | (Peng et al., 2002) |
| Firefly Luciferase | CGUACGCGGAAUACUUCGA(dTdT) | Dharmacon, Inc |

* Institute of Cancer Biology, Copenhagen, Denmark

5.8. Western blotting

Total cell lysates were prepared from cells treated with siRNA. Cells were lysed with sample buffer (20 mM TrisHCl pH8, 20 mM NaCl, 10% glycerol, 1% NP40, 10 mM EDTA, 2 mM PMSF, leupeptin 2.5 µg/ml, pepstatin 2.5 µg/ml) followed by heating to 100°C for 5 min. Protein concentration was determined by the Bradford method (BioRad, Richmond, CA); 10 µg of protein per lane were loaded on 12% SDS-PAGE minigels, and transferred to nitro-cellulose (Amersham Biosciences, Bucks, UK). Immunoblots were blocked in 5% non-fat dry milk in TBST (50 mM TrisHCl pH 7.4, 200 mM NaCl, 0.04 % Tween 20). Primary antibodies anti- α -tubulin (Sigma) (diluted 1:10,000), rabbit anti-Mdc1 (diluted 1:10,000), and anti-Nbs1 (Novus Biologicals), 100-143 (diluted 1:5,000), rabbit and Rad52 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sc-8350 (diluted 1:5,000), and mouse monoclonal antibody against DNA-PKcs (Santa Cruz Biotechnology), sc-5282 (diluted 1:5,000), mouse monoclonal anti-Mre11 (Genetex, San Antonio, TX, USA) 12D7 (diluted 1:2,000), mouse monoclonal anti-Rad50 (Genetex), 13B3 (diluted 1:4,000), mouse monoclonal E1b55K (gift from A. Levine), B-6 (diluted 1:5,000)

Secondary antibodies were goat anti-rabbit-IgG and goat anti-mouse-IgG horseradish peroxidase-labeled (DAKO, Glostrup, Denmark), 1:1,000. Secondary antibody detection was performed using ECL chemiluminescence system (Amersham Biosciences).

5.9. Real-time PCR quantification of AAV site-specific integration.

Targeted integration of AAV-Rep-GFP into AAVS1 of human chromosome 19 (chr-19) was detected by real-time PCR at virus-cellular junctions. A two-step PCR assay was established as follows. Purified genomic DNA samples (1 μ g) were preamplified (13 cycles in Applied Biosystems GeneAmp PCR System 2700), in a final volume 50 μ L in 1X polymerase buffer containing 2.5 U of Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), 200 μ M of each of the four deoxynucleoside triphosphate, 1.5 mM MgCl₂, 200 nM of primer PAAVS1 (5'-TCAGAGGACATCACGTG-3') (1593-1609nt of AAVS1 accession # S51329) and 200 nM of primer PITR (5'-TTAACTACAAGGAACCCCTA-3') previously described by (Huser et al., 2002). These primers are located outside the sequence region where the majority of published junction breakpoints have been mapped (Huser et al., 2002) Thus, most integration events should be detected. Assay conditions were as follows: 95°C for 5 min (hot start); 13 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min; and then a final elongation step of 72°C for 15 min. A real-time Taqman PCR was then performed with 2 μ L aliquots from the first PCR diluted in a final reaction volume of 25 μ L. The reaction mixture included Taq polymerase (SIGMA JumpStart Taq ReadyMix, Sigma-Aldrich, Steinheim, Germany), 1.5 mM MgCl₂, 500 nM primer PAAVS1, 500 nM primer PITR, 400 nM of Taqman

probe 6-FAM-CACTCCCTCTTCCC-MGB) complementary to AAVS1 sequence 1513-1526nt (AAVS1 accession number S51329). Amplification conditions were 50°C for 2 min, and 95°C for 10 min, and 45 cycles of 95°C for 1 min, 56°C for 1 min, and 60°C for 3 min (ABI Prism 7000 Sequence detection System). The number of integration events was calculated by linear regression of the Threshold Cycle (CT) and the concentration (expressed as number of molecules) of a standard curve of known concentrations of the plasmid pAAVS1-TR (kindly provided by R. Heilbronn, Berlin, Germany)

5.10. Cell cycle profiling

All of the cells in the plates including cells floating in the medium and released during PBS washes were collected and analyzed. Adherent cells were released from the plastic by mild treatment with trypsin. Cells were washed in twice with PBS and resuspended in PBS containing 0.1% of NP40, RNase A (2 µg/ml) and propidium iodide (5 µg/ml) were added and incubated for 30 minutes at 37°C. Samples were analyzed with a FACScan (Becton Dickinson). Data were analyzed with ModFit LT software (Verity Software House, Topsham, ME).

6. RESULTS

6.1. Interaction between recombinant Adeno-associated virus (rAAV) and cellular DNA proteins: effect on vector transduction.

The single-stranded DNA nature of recombinant AAV entails that the incoming genomes, once in the nucleus, must be converted into a transcriptionally competent double-stranded template. This process relies on the molecular machinery of the host cell.

Despite several recent advances, the molecular determinants that govern AAV genome processing are still not completely understood. Since this step represents one of the major roadblocks that limit the vector efficiency (Ferrari et al., 1996; Fisher et al., 1996), the comprehension of the biological events that determine the fate of rAAV DNA in the target host cells is fundamental for future technical improvement of AAV based gene delivery vector technology.

6.1.1. Visualization of the nuclear sites of double-stranded AAV DNA formation in live cells.

To better address the study of the kinetics of cell transduction with rAAV and, most notably, to visualize the dsDNA vector genomes in living cells, we constructed a recombinant AAV vector (AAV-LacO.14), carrying 112 Lac repressor (LacR) binding sites (LacO repeats) cloned between the viral ITRs (see Materials and Methods section 5.2); this vector was used to infect HeLa and MRC5 cell lines that stably express a GFP-LacR fusion protein with a C-terminal nuclear localization signal (HeLa/GFP-LacR and MRC5/GFP-LacR cells). The rationale of this approach is that the fluorescent sequence-specific DNA binding protein only binds its target sites when these are present in a dsDNA form, thus

allowing the dynamic visualization of dsDNA formation over time (Figure 6.1A). We found that the generation of double-stranded rAAV DNA was not diffuse throughout the nucleus but appeared restricted to specific nuclear sites, resembling the AAV foci originally detected by FISH by our group (data not shown) and other groups (Weitzman et al., 1996; Wistuba et al., 1997). Snapshots of a live imaging time courses of MRC5/GFP-LacR cells infected with AAV-LaO.14 are shown in Figure 6.1B. These foci were detected as early as 3 hours post infection as tiny bright spots within the diffuse GFP-LacR background signal inside the nucleus and then expanded into larger structures over time. The percentage of cells showing AAV foci increased during the first 24 hours post-infection in both untreated and HU-treated cells and decreased at later time points (Figure 6.1C); at all times, the percentage of cells with AAV foci was higher after HU treatment ($25.1 \pm 9.2\%$ and $11.9 \pm 0.6\%$ in HU-treated and untreated cells respectively at 24 hours).

The number of AAV-LacO.14 foci per nucleus was also determined in 3D-images from 30 to 60 nuclei observed over time (Figure 6.1D). The maximum number of foci was detected at 24 hours post-infection in HU-treated cells (23.9 ± 19.1 foci per nucleus) and at 8 hours in the untreated cells (12.1 ± 9.6). Interestingly, the number of AAV foci was only modestly increased at these times as compared to the earlier time points, most likely suggesting that their number per cell was determined very early after infection. Finally, AAV foci progressively disappeared at longer time points, even if some foci were found to persist as long as 6 days in both HU-treated and untreated cells (data not shown).

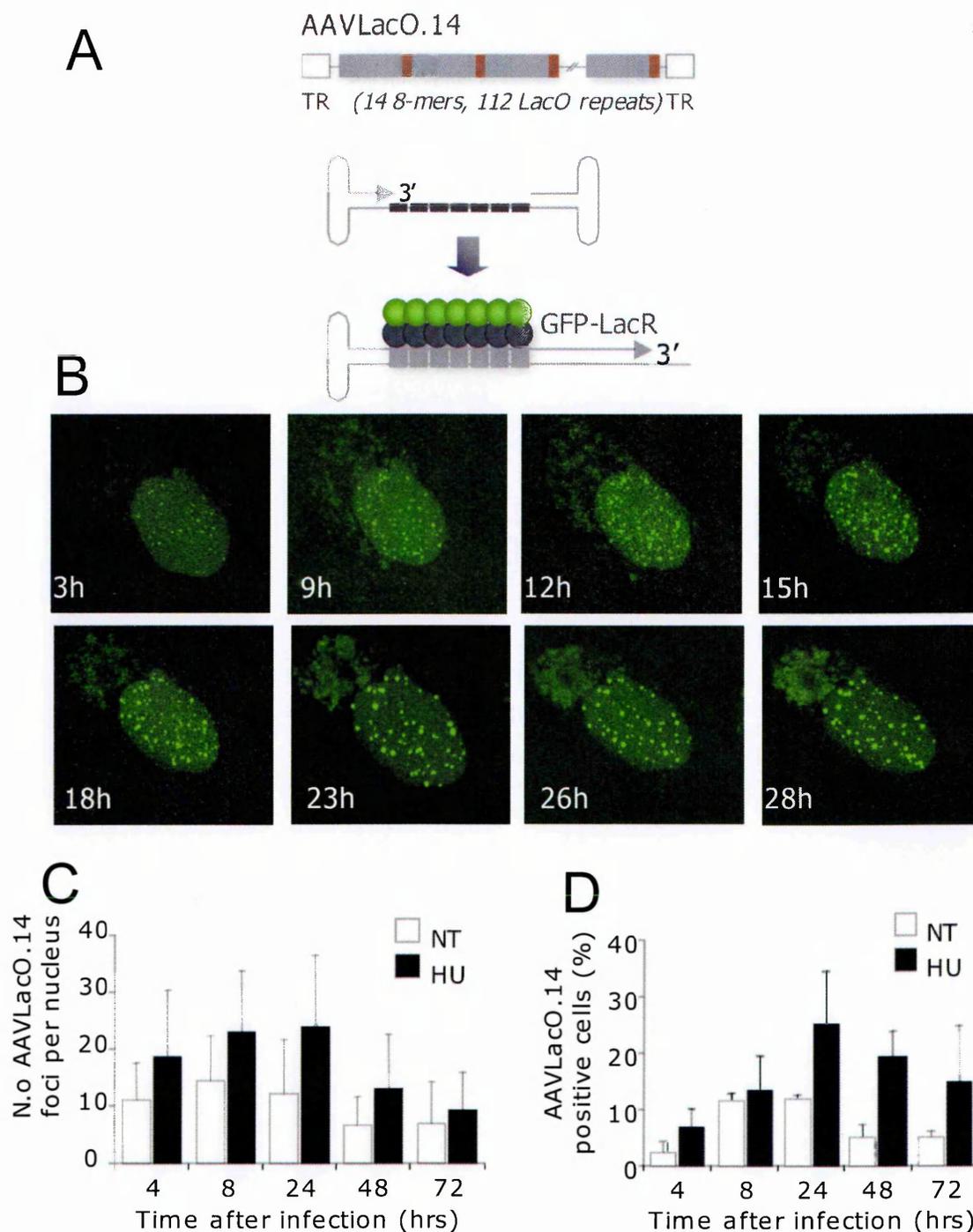


Figure 6.1. Visualization of the nuclear sites of single-stranded to double-stranded AAV-LacO.14 DNA conversion (AAV foci) in live GFP-LacR cells.

A. pAAV-LacO.14 contains the AAV ITRs flanking 14 copies of the Lac operator sequence cassette (LacO), containing a total of 112 LacR binding sites. Inside the cells, the GFP-LacR binding protein only binds the vector genome after its conversion to dsDNA.

B. Selected images from a time-lapse series of an individual MRC5/GFP-LacR cell, treated with HU and observed from 3 to 28 hr post infection. The location of AAV foci was monitored through a z-series of images. Along the z axis, nuclei were captured at 0.25 μm interval, and the final images were obtained by projection of the individual sections.

C and D. MRC5/GFP-LacR were transduced with AAV-LacO.14 either without or after treatment with HU. Live cells were analyzed at different times post-infection by counting the number of cells displaying AAV foci (**C**) and the number of foci per cell (**D**). The mean \pm s.d. of 30-60 cells per time point are shown.

6.1.2. Characterization of dsDNA recombinant AAV foci.

Detailed characterization about the fluorescence intensity and relative movement of this newly identified rAAV foci was conducted by Tiziana Cervelli (Scuola Normale di Pisa, Pisa, Italy) and is described in a coauthored manuscript submitted for publication (Cervelli, *et al*, manuscript under review, Journal of Cell Science).

6.1.3. rAAV foci form in close proximity with cellular Mre11-Rad50-Nbs1 (MRN) complex repair foci.

Given the single stranded nature of AAV genomes and the existence of regions of secondary DNA structure such as the viral ITRs within the vectors, we wanted to establish whether a relationship existed between the formation of AAV foci and the localization of cellular proteins involved in the recognition and repair of DNA damage. One of the cellular factors that are first recruited to the site of DNA damage and that can bind hairpin-structured DNA is the MRN complex, composed by the Mre11, Rad50 and Nbs1 proteins. This complex binds single-stranded and dsDNA and has a pivotal role in sensing, processing and repairing DSBs (D'Amours and Jackson, 2002; Petrini and Stracker, 2003; van den Bosch et al., 2003). To study the spatial relationship between MRN proteins and rAAV foci in the nucleus of transduced cells, we simultaneously visualized GFP fluorescence and immunostained the MRN proteins in MRC/GFP-LacR and HeLa/GFP-LacR cells at different times after transduction with AAV-LacO.14.

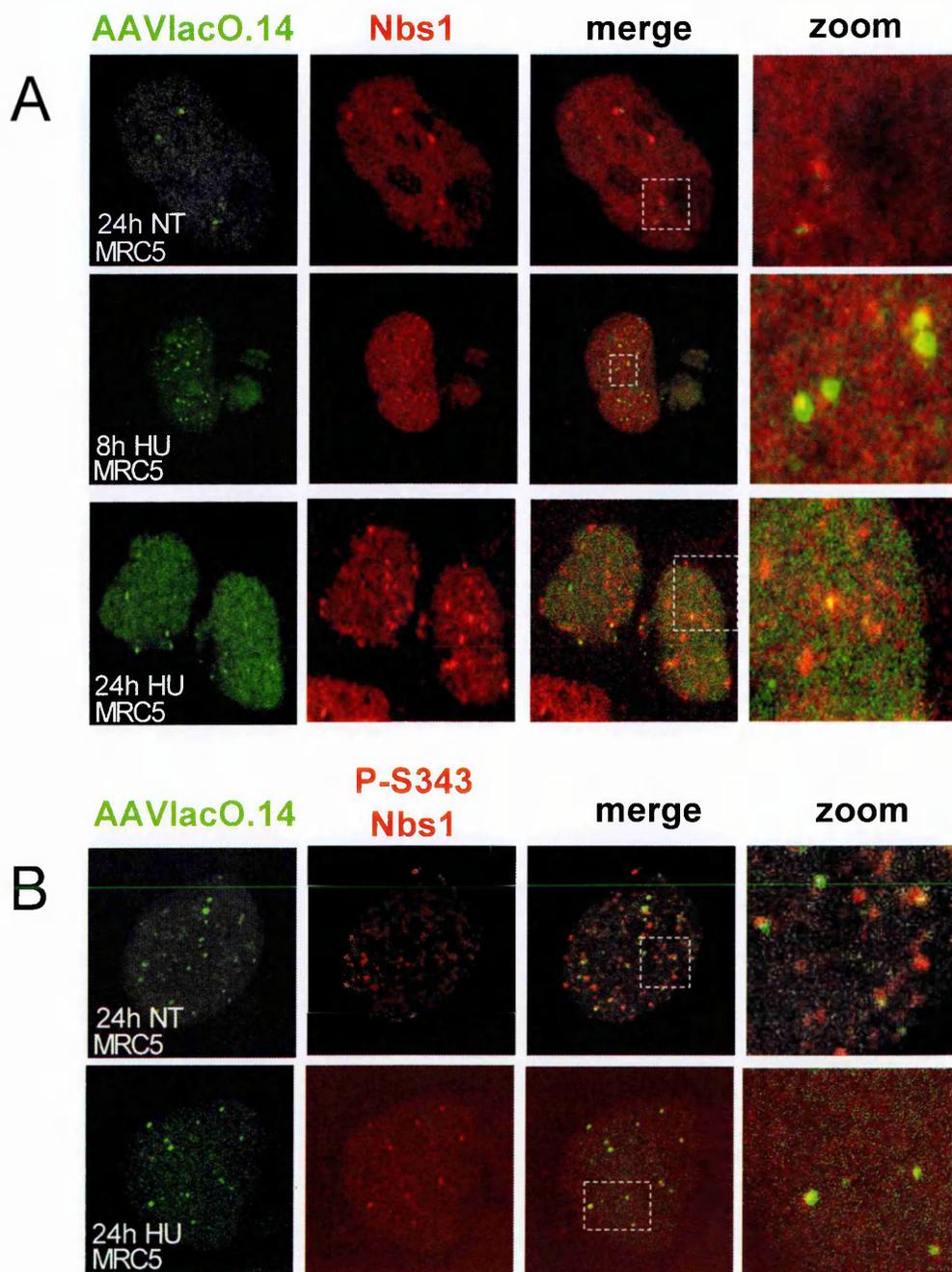


Figure 6.2. Colocalization of AAV foci Nbs1 and Phospho-Serine 343 Nbs1 foci.

MRC/GFP-LacR cells were transduced with AAV-LacO.14 and then immunostained at the indicated time points with anti-Nbs1 (A), and anti-P-S343-Nbs1 (B). HU: cells were treated with hydroxyurea (1 mM overnight) prior to AAV-LacO.14 transduction. NT: non treated

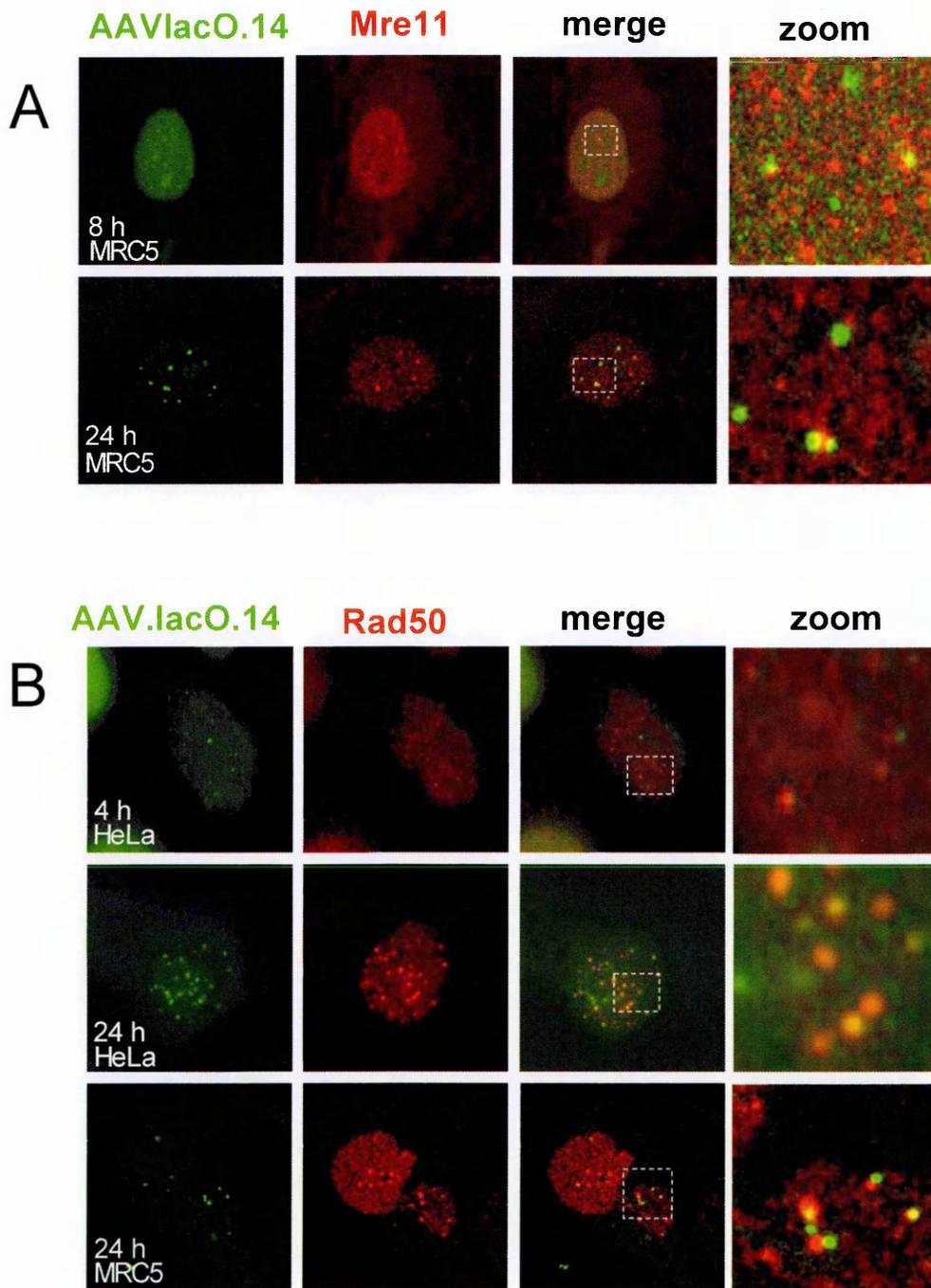


Figure 6.3. Colocalization of AAV foci with Mre11 and Rad50 foci.

Hela/GFP-LacR or MRC/GFP-LacR cells, as indicated, were treated with 1 mM HU, transduced with AAV-LacO.14 and then immunostained at the indicated time points with anti-Mre11 (A), anti-Rad50 (B). HU: cells were treated with hydroxyurea (1 mM overnight) prior to AAV-LacO.14 transduction.

In the absence of DNA damaging agents, MRN complex proteins had a predominantly diffused nuclear localization; MRN repair foci were detectable only in a few cells (<3%). The presence of repair foci in the cells not treated with HU may be associated with spontaneous and cellular DNA replication damage (Bekker-Jensen et al., 2006; Robison et al., 2004). However, when repair foci was present, we observed colocalization or close proximity of the Nbs1 foci and the AAV-Lac.O14 foci (Figure 6.2). After HU treatment, the number of cells with both AAV and Nbs1 foci was greatly increased and the two nuclear structures partially co-localize also at earlier time points after transduction (shown at 8 and 24 hours in Figure 6.2). Notably, in both untreated and HU-treated cells, rAAV foci co-localized with Nbs1 phosphorylated at serine 343, a marker of protein activation after DNA damage (Figure 6.2B) (Gatei et al., 2000; Lim et al., 2000; Wu et al., 2000).

The same visualization experiments were also performed to detect Mre11 and Rad50 proteins. In both MRC5 and HeLa cells, the vast majority of the foci formed by Mre11 and Rad50 proteins after HU treatment were found to co-localize or to be spatially juxtaposed to AAV foci at both 8 and 24 hours after transduction for Mre11 and, 4 and 24 hour after transduction for Rad50 (Figures 6.3A and 6.3B).

6.1.4. Nbs1 inhibits the formation of dsDNA rAAV foci.

In order to understand the functional relevance of the MRN colocalization with the rAAV foci, we performed silencing of Nbs1 proteins using siRNA technology. Nbs1 protein levels were decreased to less than 20% in HeLa/GFP-LacR cells transfected with an siRNA against the Nbs1 mRNA (siNbs1) (Figure

6.4A), when compared with cells treated with a control siRNA against Luciferase (siLuc) or Mock transfected cells. After effective siRNA mediated silencing of Nbs1, we infected cells with the AAV-Lac0.14 vector (see Materials and Methods section 5.4 for detailed experimental procedure) and counted the number of cells containing rAAV foci and the number of foci per cell at 16 and 24 hours post infection (p.i) with or without HU treatment.

After Nbs1 silencing, the number of cells with rAAV foci was markedly increased as compared to control cells treated with an unrelated siRNA against luciferase. This increase in the number of cells with foci was evident at both 16 h and 24 h after transduction and in the presence or absence of HU treatment (Figure 6.4C). At 24 h after transduction and in the absence of HU treatment, there were $41.08 \pm 9.93\%$ of cells with foci when treated with anti-Nbs1 siRNA, in comparison with $10.35 \pm 4.75\%$ of cells with foci when treated with the control siRNA (representative images are shown in Figure 6.4B). Of interest, the effect of HU on the number of cells with rAAV foci was less pronounced at 24 h after transduction as compared to 16 h (1.26 fold increase in the number of foci upon HU treatment at 24 h, 3.19 fold increase at 16 h), indicating that the mechanism by which HU induces rAAV foci formation is saturable when Nbs1 is knocked down.

In contrast to the number of cells showing rAAV foci, the total number of foci per cell, which had quite an ample range similar to that observed in MRC5 cells (Figure 6.1D), was less sensitive to either HU treatment or Nbs1 knock down (7.85 ± 7.78 and 4.05 ± 8.17 foci in the Nbs1 and luciferase silencing, respectively, in the absence of HU; 4.88 ± 4.35 and 3.05 ± 3.34 foci in the Nbs1 and luciferase silencing respectively, in the presence of HU at 24 h post-infection). These results reinforce the notion that both Nbs1 and HU act by increasing the efficiency

and kinetics of rAAV formation but do not significantly alter the frequency at which these structures are formed and that Nbs1 play an inhibitory role at the level of single-stranded to dsDNA conversion and accumulation into the rAAV foci.

6.1.5. Nbs1 inhibits rAAV transduction.

HeLa and MRC5 cell lines were infected with a rAAV vector coding for β -galactosidase (AAV-LacZ) and transduction efficiency was determined by β -galactosidase enzymatic activity. In both cases, HeLa and MRC5 cells in which the Nbs1 gene was silenced were significantly more permissive to AAV transduction at all tested MOIs (Figure 6.5). Cells treated with HU showed a higher level of transduction when compared with control siLuc transfected cells. In particular, in the case of MRC5 cells, there was a gradual increase in the relative difference or fold increase between cells where Nbs1 was silenced in comparison with control cells at higher MOIs. This observation indicates that Nbs1 plays an inhibitory role at the level rAAV transduction and that permissivity to functional AAV transduction correlates with the number of cells with AAV foci, and thus with the level of dsDNA genomes inside the cell's nucleus.

6.1.6. Nbs1 inhibitory role on rAAV transduction requires ATM function.

AT5 cells, that do not express a functional ATM protein, are known to be highly permissive to rAAV transduction and thus to respond only minimally to hydroxyurea treatment (Zentilin et al., 2001) (see also Figure 6.6A). We hypothesized that the high permissivity to rAAV of ATM(-/-) cells, that we previously described, may be due to an intrinsic inability of these cells to activate

cellular target proteins downstream ATM, like Nbs1, that may exert an inhibitory effect on AAV transduction. In AT5 cells, Nbs1 silencing had minimal or no effect on the efficiency of rAAV transduction, using a broad range of MOIs of the AAV-LacZ vector (Figure 6.6B). These results suggest that the negative role of Nbs1 protein on AAV transduction might require functional ATM activity.

6.1.7. Adenovirus E4orf6 and E1B55k mediated degradation of MRN complex increases rAAV transduction.

Productive AAV replication and rAAV transduction requires an unrelated helper virus, such as Adenovirus. Adenovirus E1B55k and E4orf6 proteins are known to enhance rAAV vector transduction and wild-type AAV replication by increasing the rate of conversion of the AAV genomes from single-stranded to dsDNA (Ferrari et al., 1996; Fisher et al., 1996), although the mechanism is unclear. It has been recently shown that E1B55k and E4orf6 form a complex that possess ubiquitin ligase activity in conjunction with cellular proteins (Harada et al., 2002; Querido et al., 2001) to promote degradation of cellular p53 and MRN complex (Carson et al., 2003; Stracker et al., 2002). Here we show that adenovirus E1B55k/E4orf6 mediated degradation of MRN complex correlates with augmentation of rAAV transduction.

We used a recombinant adenoviral vector coding for the E4orf6 to infect a series of HeLa stable cell clones expressing: the wild-type E1B55k and GFP-LacR (HeLa wtE1B55k/GFP-LacR); a mutant form of E1B55k that is unable to associate with E4orf6 and to effectively degrade the MRN complex and GFP-LacR (HeLa H354/GFP-LacR); and only GFP-LacR (HeLa/GFP-LacR).

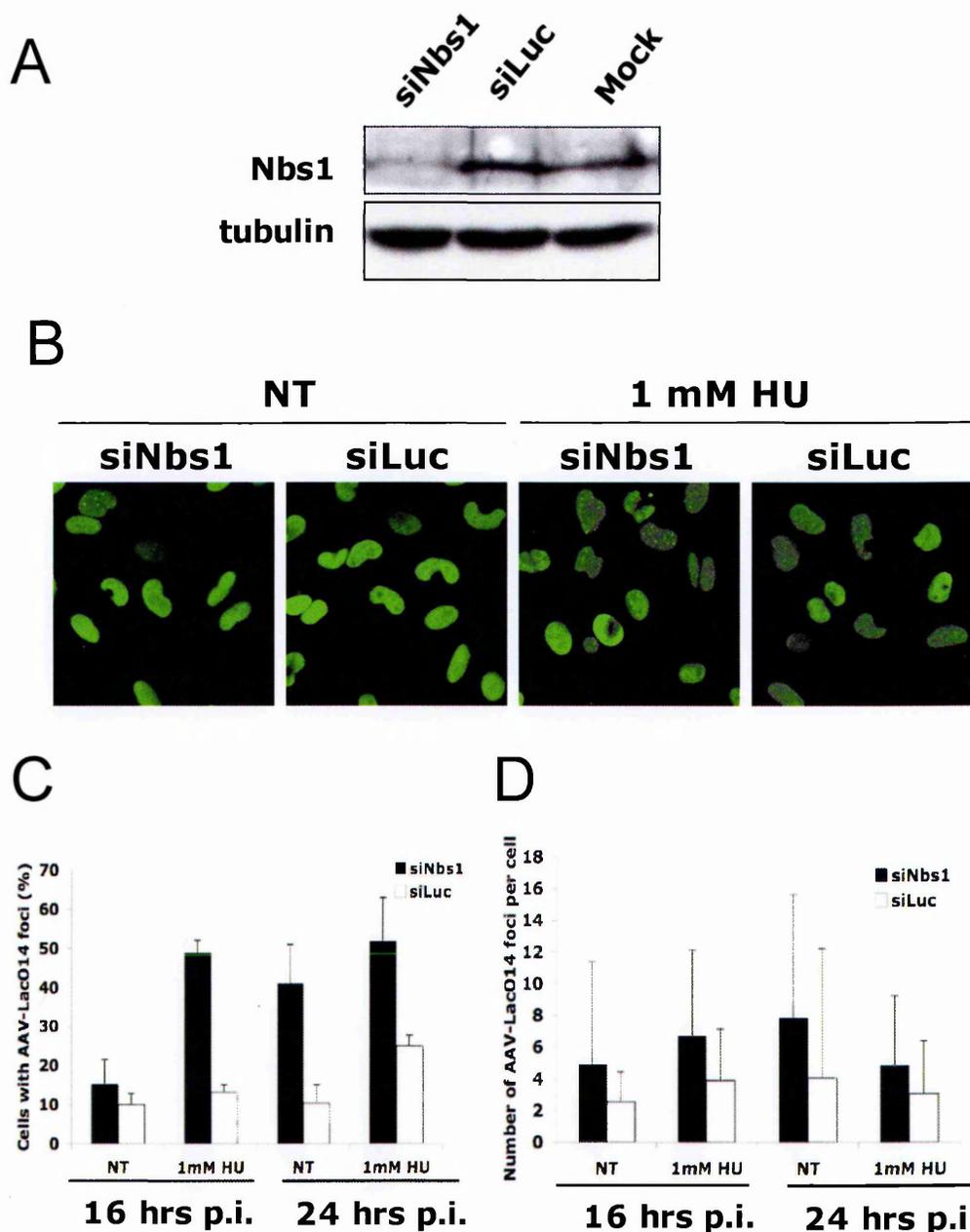


Figure 6.4. Silencing of Nbs1 increases rAAV foci formation.

A. Western blotting showing the levels of Nbs1 in HeLa cells after treatment with siRNAs against Nbs1 or luciferase (Luc) at 60 h after siRNA transfection (corresponding to 24 h p.i.). The western blotting against tubulin is used as a loading control.

B. Representative images of cells treated with anti-Nbs1 or anti-Luc siRNAs followed by transduction with AAV-GFP. The images were taken at 24 h p.i. in the absence (NT) and after HU treatment.

C. Percentage of HeLa cells with detectable AAV-LacO.14 foci after silencing of Nbs1 or treatment with control siRNA against Luc.

D. Number of AAV-LacO.14 foci per cell after silencing of Nbs1 or treatment with control siRNA against Luc.

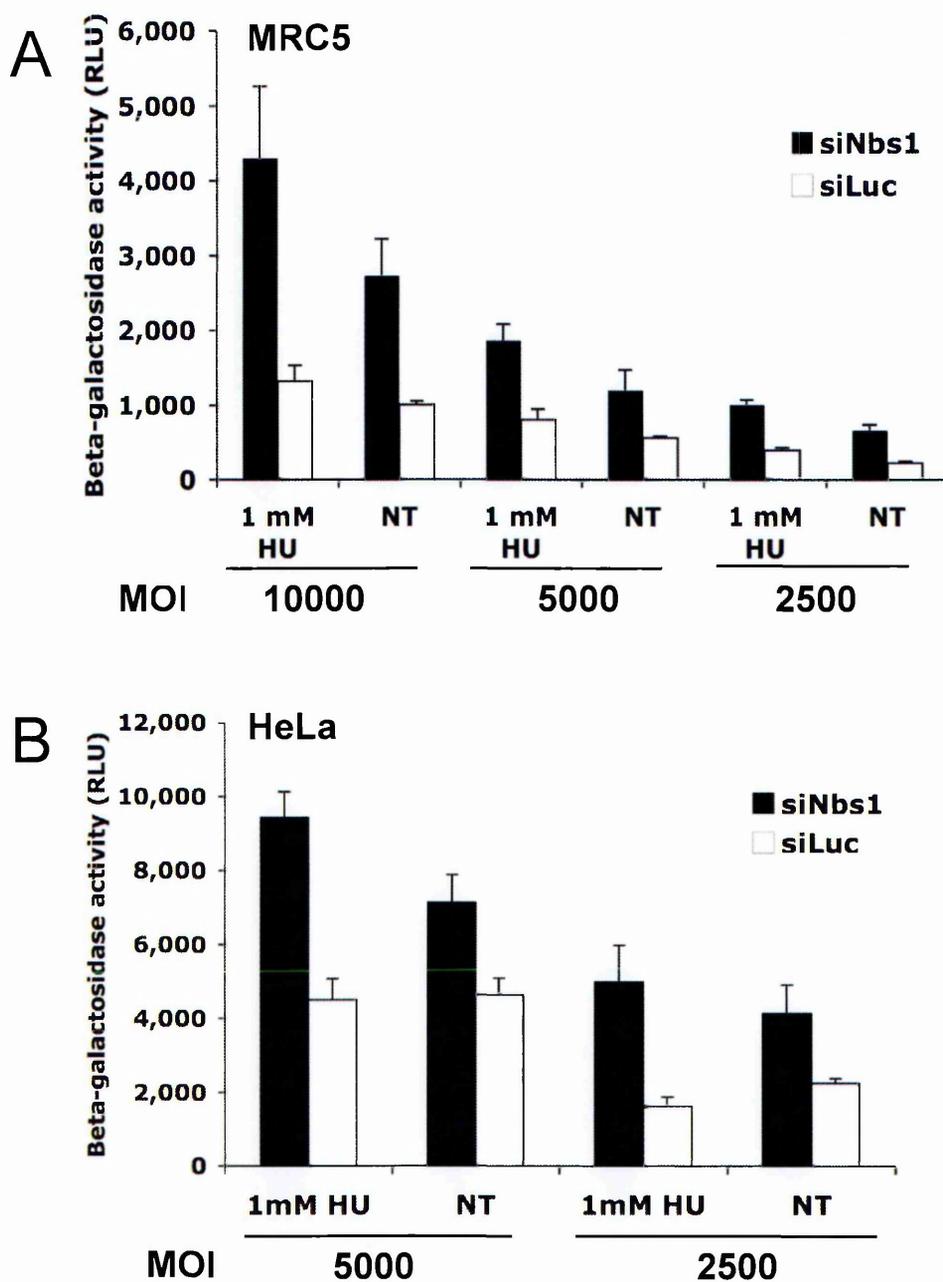


Figure 6.5. Silencing of Nbs1 increases rAAV transduction.

A. β -gal enzymatic activity measured as relative light units (RLU) in lysates from MRC5 cells after treatment with siRNAs against Nbs1 (siNbs1) or against the control gene luciferase (siLuc), followed by transduction with AAV-LacZ.

B. Same as in panel A in HeLa cells.

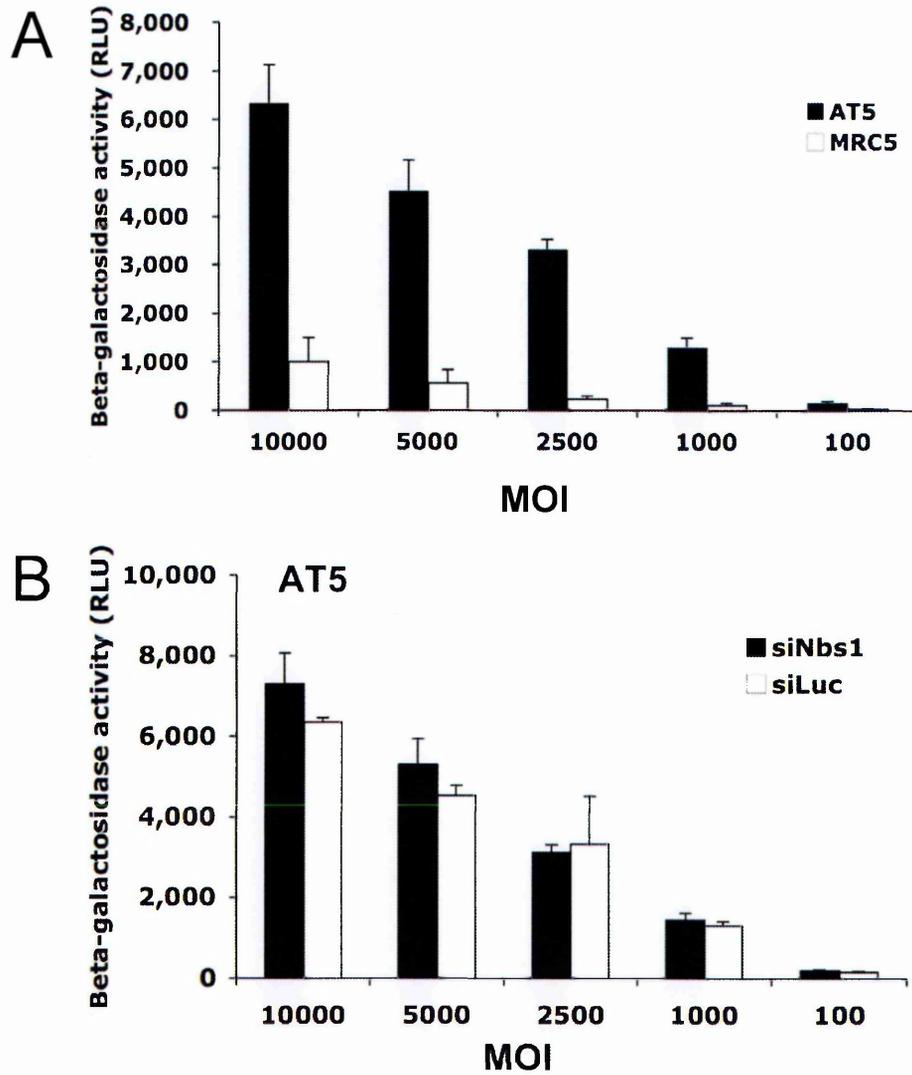


Figure 6.6. AT5 cells are more permissive to rAAV and Nbs1 inhibition role requires ATM function.

A. β -gal enzymatic activity measured as relative light units (RLU) in lysates from AT5 and cells after transduction with AAV-LacZ.

B. β -gal enzymatic activity measured as relative light units (RLU) in lysates from AT5 cells after treatment with siRNAs against Nbs1 (siNbs1) or against the control gene luciferase (siLuc), followed by transduction with AAV-LacZ.

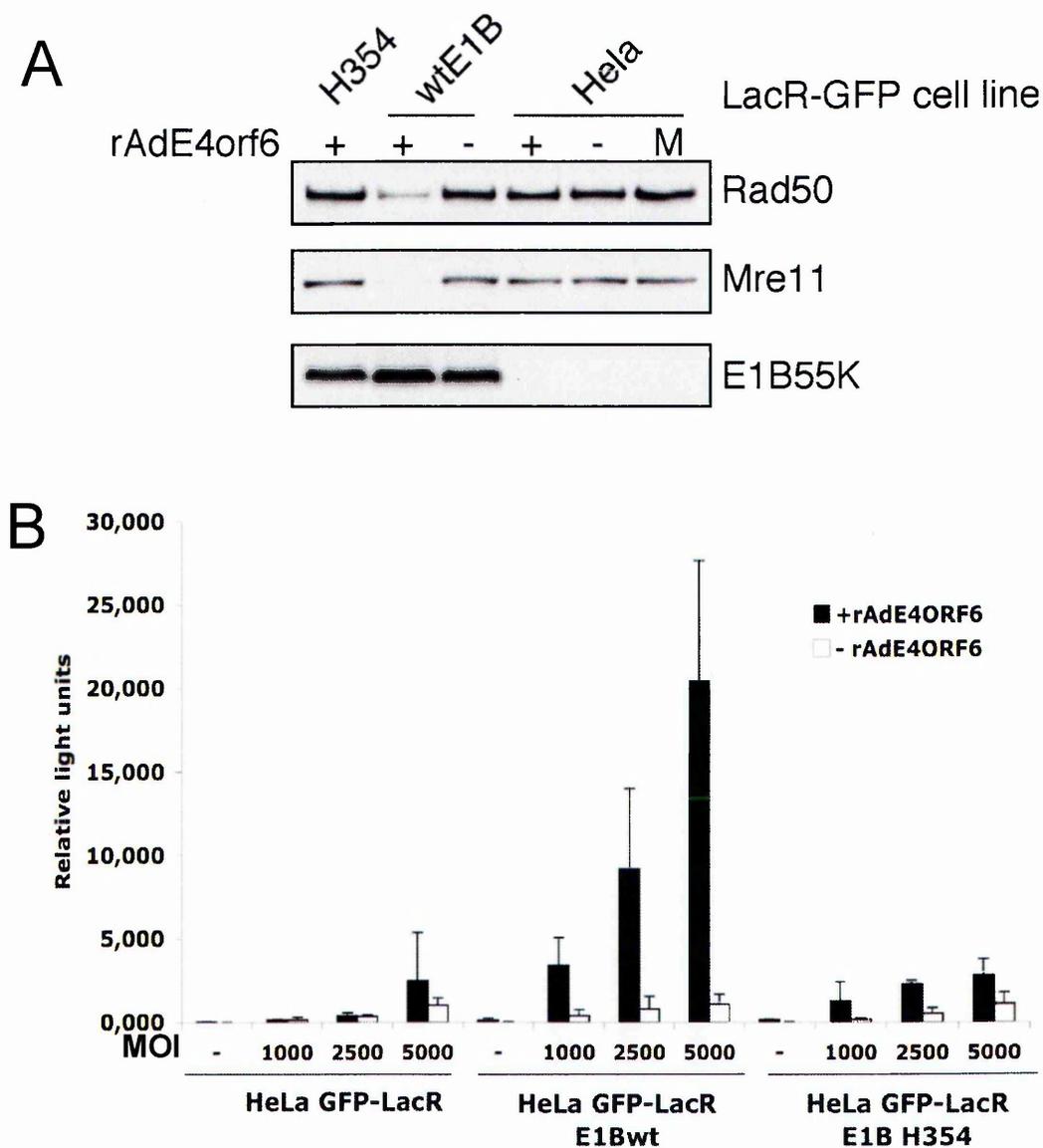


Figure 6.7. Adenovirus E4orf6/E1b55k mediated degradation of MRN complex increases rAAV transduction.

A. HeLa-derived cell lines expressing GFP-LacR or GFP-LacR with E1b55K or mutant H354 proteins were analyzed by western immunoblotting for E1b55K expression and degradation of the Mre11 complex in the presence of E4orf6.

B. β -gal enzymatic activity measured as relative light units (RLU) in lysates HeLa-derived cells lines expressing GFP-LacR or GFP-LacR with E1B55k or mutant H354 after transduction with AAV-LacZ.

As expected, degradation of Mre11 protein and partial degradation of Rad50 was only observed when wtE1B55k and E4orf6 proteins were expressed in the same cells (Figure 6.7A). When these cells were additionally infected with rAAV-LacZ and β -galactosidase activity was measured, we observed a dramatic increase in the level of transduction in the cells where MRN complex was degraded (Figure 6.7B). This increase in transduction was not observed in cells infected with rAd-E4orf6 and expressing the H354 mutant E1B55k. These results correlate Mre11 degradation with enhanced rAAV transduction, and suggest that the Mre11 complex negatively impacts rAAV transduction. A more detailed characterization of this correlation is shown in a coauthored manuscript with the M.D. Weitzman group (Schwartz *et al*, manuscript under revision, EMBO reports).

6.1.8. E1B55k/E4orf6 mediated degradation of MRN complex correlates with increased number of cells with dsDNA rAAV foci.

In order to see if transduction efficiency of rAAV correlates with the formation of double stranded rAAV DNA genomes, we treated wtE1B/GFP-LacR, H354/GFP-LacR, and GFP-LacR HeLa cell clones with rAd-E4orf6 and then infected them with AAV-LacO14 vector. As expected, cells expressing simultaneously wtE1B55k and E4orf6 showed an increase in the number of cells with rAAV foci, that represent sites of accumulation of vector dsDNA, when compared with the same cell lines not infected with rAd-E4-orf6 (from $7.9 \pm 0.8\%$ to $59.3 \pm 5.6\%$). HeLa H354/GFP-LacR cell line infected rAdE4orf6 showed a higher number of cells with foci in comparison to the same cell line not infected with rAd (from $6.4 \pm 1.3\%$ to $19.5 \pm 5.8\%$) (Figures 6.8A and 6.8B). On the

contrary, in the cell line expressing just GFP-LacR we detected only a basal number of cells with AAV foci, that were about $4.4 \pm 2.0\%$ and $3.8 \pm 1.3\%$ in the presence or absence of infection with rAdE4orf6 respectively. These results together show, that E1B55k/E4orf6-mediated degradation of the MRN complex increases rAAV foci formation and transduction, suggesting again that the MRN complex may play an inhibitory role at the level of single-stranded to dsDNA rAAV genome conversion. E1B55k/E4orf6-mediated degradation of the MRN complex did not affect in a statistically significant manner the number of rAAV foci per cell (Figure 6.8C).

6.1.9. AAV foci form in close proximity of Mdc1 foci.

The Mdc1 protein is known to control cellular responses to DNA damage, in part by interacting with the MRN complex and, more specifically, by mediating the transient interaction of Nbs1 with DSBs and its phosphorylation by ATM (Goldberg et al., 2003; Lukas et al., 2004; Stewart et al., 2003; Xu and Stern, 2003). In our experimental settings, AAV-LacO.14 foci were also found in close proximity with the foci at which the Mdc1 protein accumulates, in both untreated cells and in cells treated with camptothecin before infection (Figure 6.9A). This colocalization suggests a possible role of Mdc1 in the recruitment of MRN complex to the site of accumulation of rAAV dsDNA.

6.1.10. Silencing of Mdc1 decreases rAAV transduction.

In order to quantify the extent of rAAV transduction in the absence of the Mdc1 protein, we took advantage of the availability of stable HeLa cell clones containing retroviral vectors expressing a short-hairpin RNAs (shRNA) against

Mdc1 or against a control protein LacZ (Lukas et al., 2004). These cells were infected with a rAAV viral vector coding of the EGFP gene (AAV-GFP) and the efficiency of transduction was measured by FACS analysis. The cell clone in which Mdc1 was silenced was significantly more permissive to AAV-GFP transduction, both in the absence or following HU treatment (Figure 6.9C). Similar results were obtained when HeLa cells were transiently transfected with synthetic siRNA against Mdc1 (Figure 6.9E). The complete silencing of the Mdc1 protein was obtained in both cases. (Figures 6.9B and 6.9D). Again, the increase in rAAV transduction after Mdc1 silencing is in concordance with the idea that Mdc1 may play a role in the inhibitory action of the MRN complex at the level of rAAV transduction.

6.1.11. Rad52 silencing results in decreased rAAV transduction efficiency.

Previous results from our laboratory indicated that the protein Rad52 binds to rAAV genomes and that Rad52 (-/-) fibroblasts are less permissive than wild type to rAAV transduction (Zentilin et al., 2001). In order to deeply investigate the role of this protein in determining the fate of rAAV genomes inside the infected cells, we transfected HeLa cells with and siRNA against Rad52. Western blot analysis revealed that 70-80% of reduction of the protein was obtained 48 hour after siRNA transfection (inset of Figure 6.10A). Under these conditions, cells treated or untreated with HU, showed a maximum of 2,1 fold reduction in the β -galactosidase activity at a MOI of 10,000 vgp/cell, when compared with the cells transfected with the control siRNA against Luciferase (Figure 6.10A). Contrary to the effect observed after silencing of Nbs1 and Mdc1, Rad52 silencing resulted in a significant decrease in vector transduction, this

independently of HU treatment. This observation is in concordance with the results obtained previously and suggests a possible involvement of Rad52 protein in the processing or stabilization of transcriptionally active rAAV genomes.

6.1.12. DNA-PKcs silencing results in decreased rAAV transduction efficiency.

DNA-PKcs, a key protein in the process of double-strand break repair by non-homologous end-joining, has been also implicated in the determination of the molecular fate of rAAV genome *in vivo*. Studies of gene delivery on mouse muscle have shown that, in the presence of DNA-PKcs, rAAV DNA form circular episomes, while in its absence, rAAV genomes remain as linear monomers or form linear concatamers (Song et al., 2001). Nevertheless, in these studies the levels of transgene expression in this system remained unchanged over time. In order to better characterize the possible role of DNA-PKcs in rAAV transduction, we transfected a specific siRNA in HeLa cells obtaining more than 80% reduction of the DNA-PKcs protein at 48 hours after treatment (inset of Figure 6.10B). Then, these cells were either treated or not with HU and infected with an AAV-LacZ vector. Interestingly, a significant reduction in vector transduction was only observed when cells were treated with HU. On the contrary, no statistically significant differences were observed between DNA-PKcs silenced and control cells when HU was not added (Figure 6.10B). These results suggest a possible participation of DNA-PKcs in rAAV processing in HeLa cells but only in the presence of HU induced cell damage.

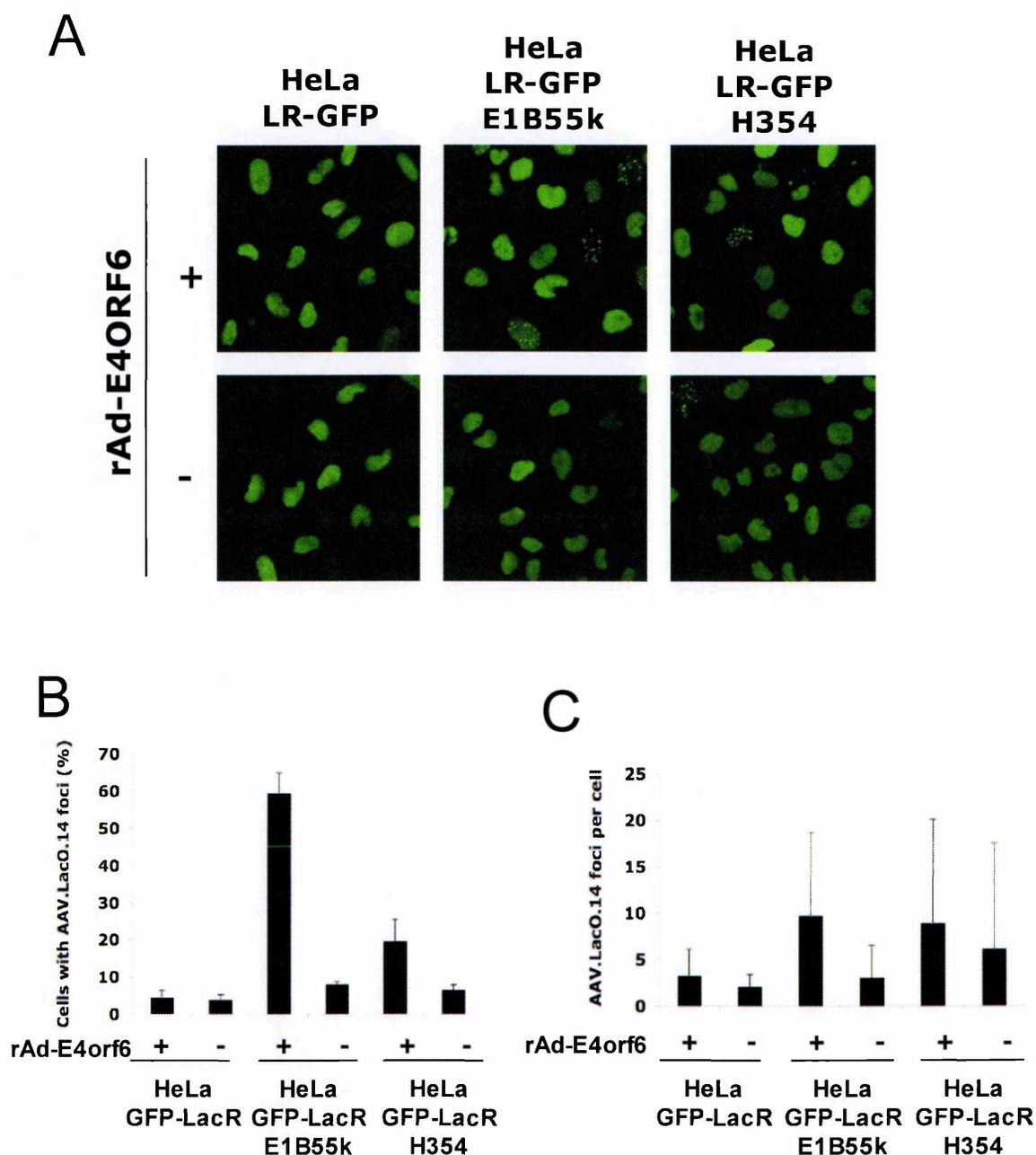


Figure 6.8. E4orf6/E1B55k mediated degradation of MRN complex correlates with an increase in the number of cells with rAAV foci but not the number of foci per cell.

A. HeLa cell lines expressing GFP-LacR alone or with E1b55K or H354 mutant were, when indicated, infected with rAd-E4orf6 (MOI 25) for 24 hrs before super-infecting with AAV-LacO.14 (MOI 400) for another 16 hrs. Cells were fixed and analyzed by confocal microscopy for rAAV-LacO.14 foci.

B. Quantification of the number of cells with AAV-LacO14 foci in GFP-LacR E1b55K/H354 cell lines previously infected, when indicated, with rAdE4orf6. For each treatment, the average of 3 groups of at least 120 cells each is presented. Error bars represent standard deviation of the mean.

C. Same as panel B but quantification of the number of foci per cell.

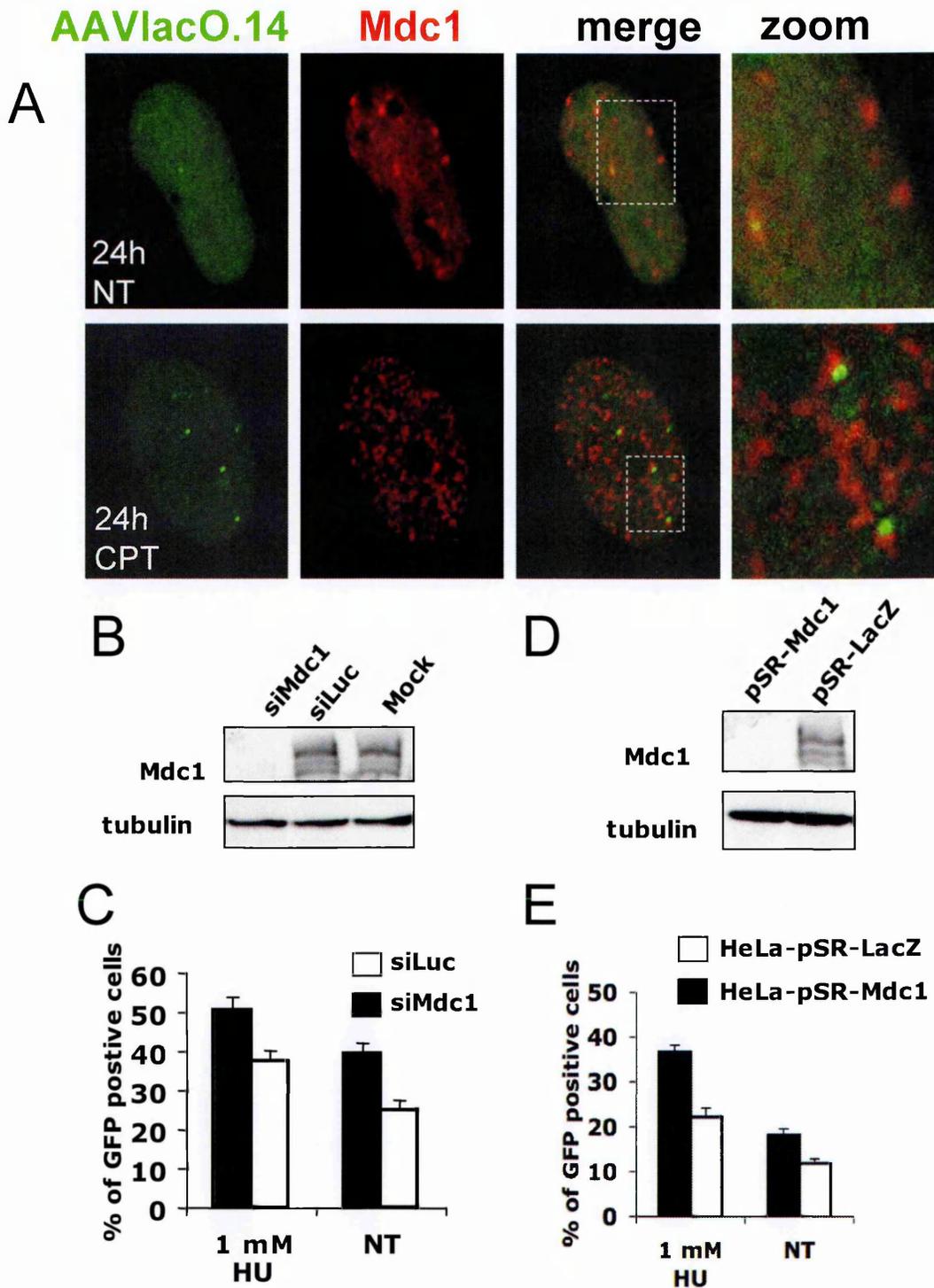


Figure 6.9. Colocalization of AAV foci with Mdc1 foci and its silencing increases rAAV transduction

A. MRC/GFP-LacR cells were transduced with AAV-LacO.14 and then immunostained at 24 hour p.i. with anti Mdc1 antibody. CPT: cells were treated with camptothecin (1 nM for 6 h) prior to AAV-LacO.14 transduction.

B. Levels of Mdc1 protein in the pSR-Mdc1 and pSR-LacZ HeLa derived cell clones.

C. Flow cytometry analysis of stable HeLa cell clones containing retroviral vectors expressing shRNAs against Mdc1 (pSR-Mdc1) or β -galactosidase (pSR-LacZ) after transduction with AAV-GFP.

D. Levels of Mdc1 protein 60 h after siRNA transfection (24 h after AAV-GFP transduction).

E. Flow cytometry analysis of HeLa cells transfected with siRNAs against Mdc1 or luciferase.

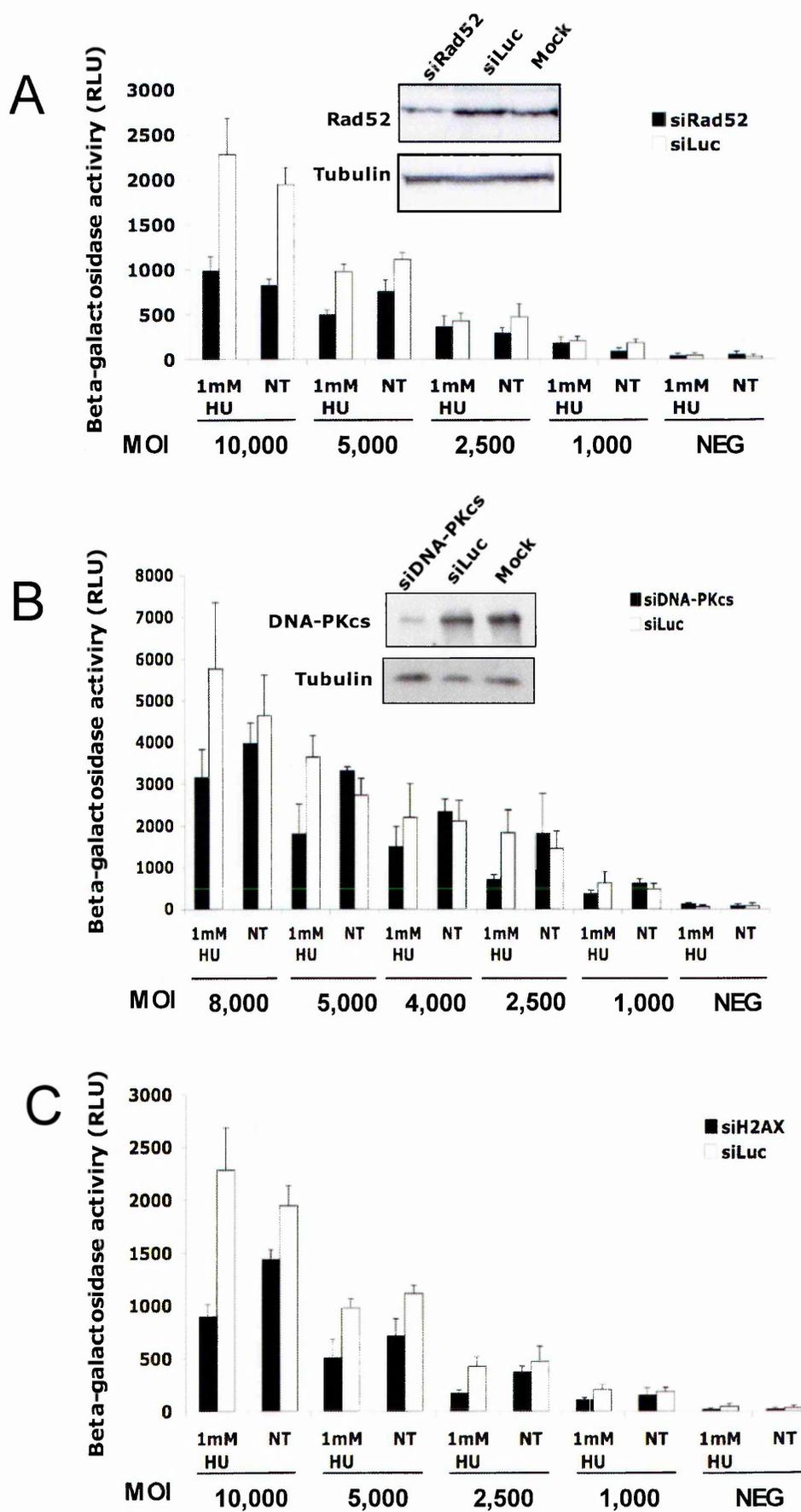


Figure 6.10. rAAV transduction after Rad52, DNA-PKcs and H2AX siRNA mediated silencing.

A. β -gal enzymatic activity measured as relative light units (RLU) in lysates from HeLa cells after treatment with siRNAs against Rad52 (siRad52) or against the control gene luciferase (siLuc), followed by transduction with AAV-LacZ (MOIs of 10,000, 5,000, 2,500, 1,000, or non-infected – NEG). Where indicated, cells were treated with hydroxyurea (overnight).

B. Same as panel **A** with siRNA against DNA-PKcs (siDNA-PKcs) at MOIs of 8,000, 5,000, 4,000, 2,500, 1,000, or non-infected – NEG)

C. Same as panel **A** with siRNA against H2AX (siH2AX)

6.1.13. H2AX silencing results in decreased rAAV transduction efficiency.

We silenced histone H2AX in HeLa cells by means of siRNA technology, before infecting them with an AAV-LacZ vector. In this case we observed results similar to those obtained after silencing DNA-PKcs. A significant reduction in transduction efficiency was observed specifically in the cells that were treated with HU (Figure 6.10C). In cells that were not treated with HU there was only a slight reduction in rAAV transduction, with respect to the cells transfected with the control siRNA against Luciferase, but only at infection MOI of 10,000 vgp/cell,. Decreasing the levels of H2AX may impair the capacity of the cell to elicit a cellular response to DNA damage (Lukas et al., 2004). These results suggest that HU treatment in cells with decreased levels of H2AX or DNA-PKcs may induce the activation of pathways that play an inhibitory role on rAAV at the level of transduction.

6.2. Interaction between AAV Rep containing recombinant Adeno-associated virus (rAAV) and cellular DNA machinery: effect on vector transduction and site-specific integration.

In the absence of a helper virus infection, wild-type AAV, as well as viral vectors containing the AAV Rep gene, mainly integrate in a site-specific fashion in human cells (McCarty et al., 2004). Both AAV DNA replication and site-specific integration require the large nonstructural Rep proteins (Rep68/78) and specific motifs within the viral ITRs. The cellular proteins participating in the process of Rep-mediated site-specific integration and the molecular mechanism of integration itself are still largely elusive. In this task we will explore the

hypothesis that the process might involve the cellular proteins that participate in the cellular DNA repair.

6.2.1. Establishment of a quantitative real-time PCR procedure for the determination of rAAV site-specific integration.

We have established a highly sensitive method to quantify the events of site-specific integration of AAV DNA into chromosome 19q13.3qter, in the region named AAVS1 (Linden et al., 1996a). The system is based on a two-step, real-time quantitative PCR assay with primers located in the AAVS1 region on Ch19q13.3qter and in the AAV ITR, as originally described by Huser, *et al.* (2002). Only the samples that contain the flanking sequences of the 3' ITR of the virus and a downstream sequence of the AAVS1 region are amplified in the first PCR reaction and then quantified using a TaqMan probe in the second PCR reaction (schematic representation shown in Figure 6.11A). Standardization of the protocol was carried out using control plasmid pAAVS1-ITR, constructed for this purpose, and genomic DNA extracted from a cell line containing site-specifically integrated AAV genomes. The developed technology showed a wide range of linearity in the determination of the number of AAV Rep-dependent site-specific integration events from 45 to 45×10^5 copies/ μg of genomic DNA, as shown in Figure 6.11B. In order to rule out the possibility of unspecific amplification of rAAV and genomic sequences in the absence of integration at the AAVS1 locus, we transfected HeLa cells with a plasmid coding for the Rep68 proteins or a control plasmid, and then infected these cells with the AAV-GFP (not expressing Rep) viral vector, as already suggested by others (Huttner et al., 2003). As expected, PCR amplification was observed only in the infected cell samples where Rep68 protein was expressed; mock infected cells or samples

infected with the AAV-GFP alone did not show any detectable amplification (Figure 6.12A).

Site-specific AAV integration requires the presence of the wtAAV Rep68 or 78, proteins that are typically not included in the rAAV vector used in gene therapy and many biotechnological applications. We constructed a rAAV viral vector that contains the wild-type Rep open reading frame (ORF) and the EGFP gene in the place of the Cap ORF (AAV-Rep-GFP) (see Materials and Methods for detailed explanation of the construct). In this way we can evaluate the level of vector transduction and site-specific integration in the same samples.

Using this technology, we demonstrated that the AAV-Rep-GFP vector DNA integrated site-specifically into the host cell DNA at the AAVS1 locus at frequencies that were comparable with those of the wt virus (6-7% of cells with site-specific integration) (Huser et al., 2002). The integration frequency was observed to increase in a dose-dependent manner at increasing MOIs (Figure 6.12B). Non-detectable integration events were observed in the absence of AAV-Rep-GFP or after infection with AAV-GFP.

6.2.2. Silencing of Nbs1 increases frequency of Rep-dependent site-specific integration.

We evaluated the transduction efficiency of AAV-Rep-GFP in HeLa cells after transfection with siRNAs against either Nsb1 or Luciferase in the presence and absence of HU treatment.

Interestingly, we observed a small but statistically significant increase in transduction of AAV-Rep-GFP at a MOI of 5,000 vgp/cell with or without HU treatment (from $29.2 \pm 1.6\%$ to $32 \pm 0.7\%$ after HU, and from $22.8 \pm 0.2\%$ to $28.9 \pm 2.3\%$ in the absence of HU) (Figure 6.13A); this in agreement with the data

already described. A remarkable finding was observed when we looked at the levels of vector integration at the AAVS1 region. We detected a dramatic increase in the level of site-specific integration after silencing of Nbs1 at both MOI of 5,000 (8.3 fold) and 1,600 vgp/cell (15.8 fold) (Figure 6.13B). Interestingly, the Rep-dependent AAV site-specific integration appears to more closely rely on the presence of the Nbs1 protein than the transduction process. Nevertheless, Nbs1 exerts an inhibitory role at both levels.

6.2.3. Silencing of Mdc1 increases frequency of Rep dependent site-specific integration.

The same experimental procedure was used to evaluate the involvement of the Mdc1 protein in the process of Rep-mediated AAV site-specific integration. HeLa cells were transfected with the siRNAs against Mdc1, or Luciferase as control, and then infected with AAV-Rep-GFP vector in the presence and absence of HU treatment. As expected, silencing of the Mdc1 protein determined an increase in vector cell permissivity, measured as percentage of infected cells expressing the GFP reporter gene (from 32.8 ± 1.0 to 37.7 ± 0.7 with HU treatment at MOI=5,000, from 22.8 ± 0.2 to 25 ± 3 without HU treatment at MOI=5,000, and from 7.6 ± 0.4 to 13.0 ± 0.1 with HU treatment at MOI=1,600). Also in this case, the effect of Mdc1 silencing was slightly blunted by the presence of the Rep protein (compare figure 6.14A and 6.9C). Nevertheless, similarly to what observed after Nbs1 silencing, Mdc1 silencing resulted in a dramatic increase (39.4 fold increase at MOI=5,000 and 2,649 fold increase at MOI=1,600) in the number of integration events at the AAVS1 locus (Figure 6.14B). All these data suggest the Mdc1 may inhibit AAV transduction in the presence of Rep protein but more remarkably it inhibits AAV site-specific integration.

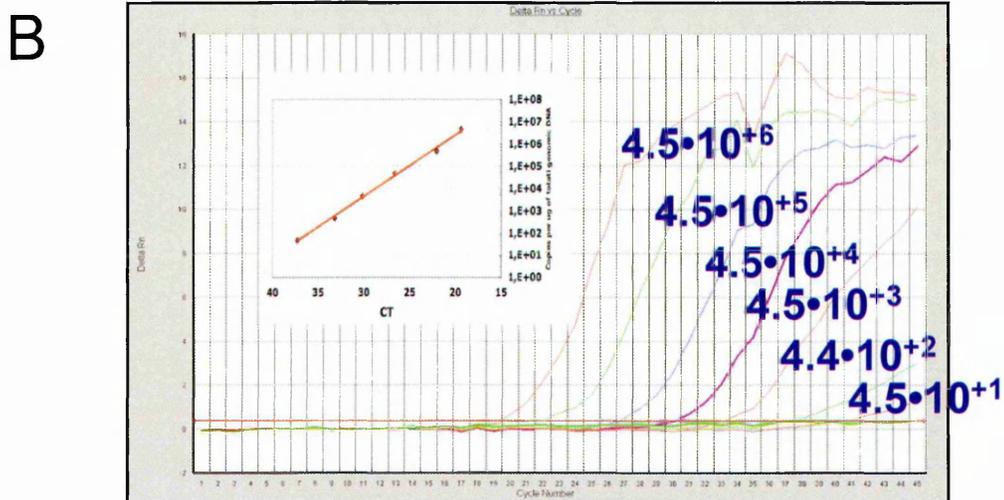
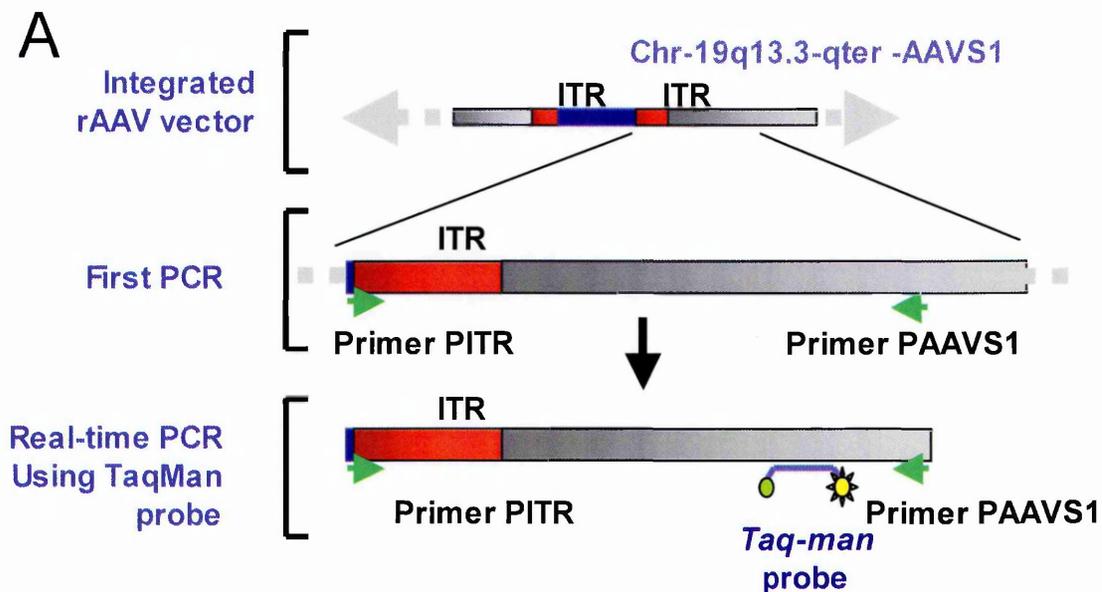


Figure 6.11. Establishment of real-time PCR for the quantification of AAV site-specific integration: Real-time PCR quantification wide range of linearity of detection in the amplification of pAAVS1-TR

A. Schematic representation of the real-time PCR quantification experimental procedure.

B. Cycle number versus ΔR_n from a representative experiment of Taqman real-time PCR amplification of pAAVS1-TR plasmid serial dilutions (from 45×10^5 copies/ μg of genomic DNA). Insert: Scattered plot of the number of copies per μg of genomic DNA as a functions of the Cycle of threshold (CT). The number of copies is represented in logarithmic scale. Minimum scores fitting curve is plotted in red.

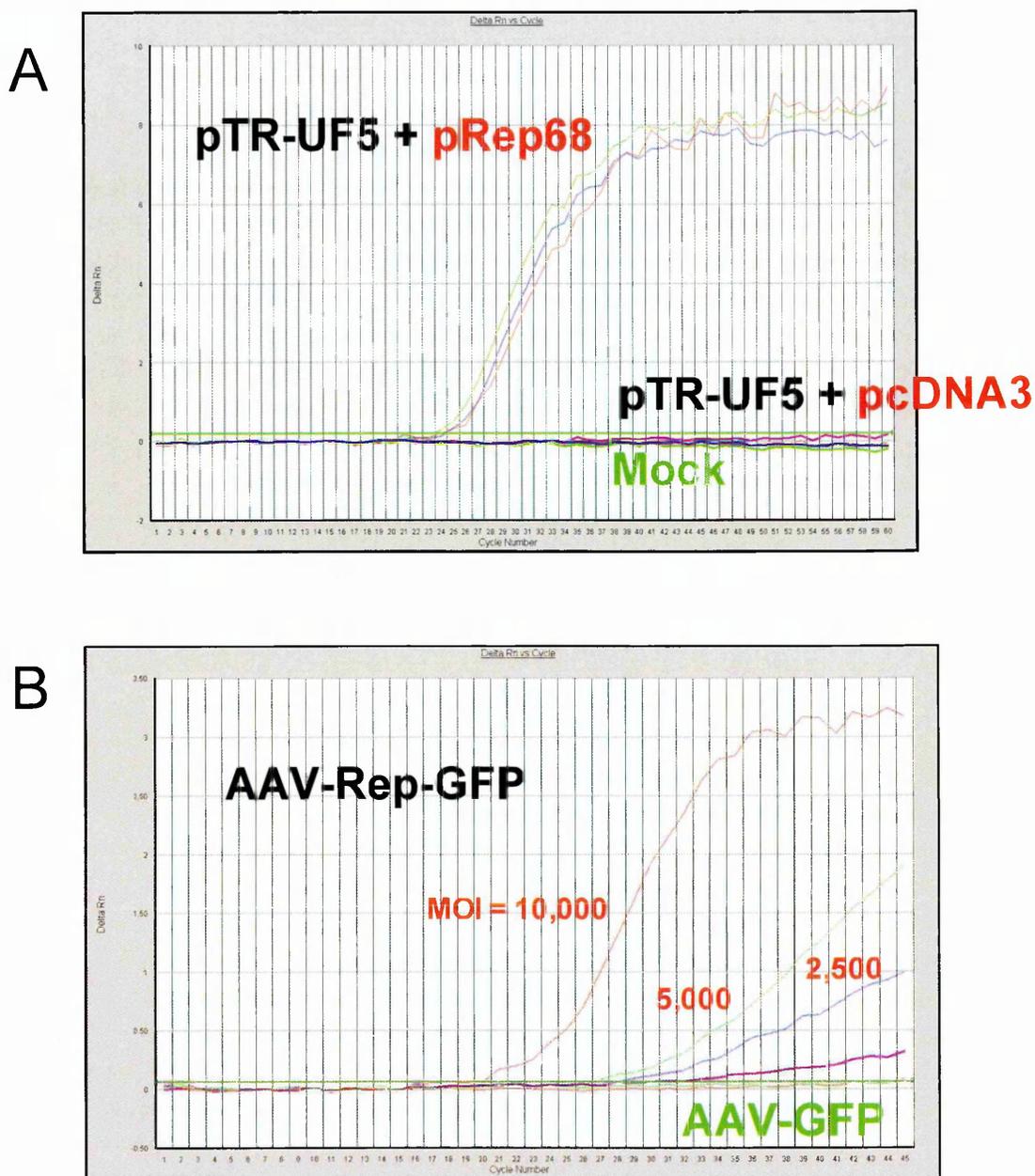


Figure 6.12. Establishment of real-time PCR for the quantification of AAV site-specific integration: PCR Amplification of AAV and AAVS1 junctions only occur when Rep is provided.

A. Cycle number versus Delta Rn from a representative experiment of Taqman real-time PCR amplification genomic DNA isolated from HeLa cells 48 hrs after transfection with pTR-UF5 (AAV-GFP) together with an AAV Rep68 expression plasmid or with the control plasmid pcDNA3, or Mock infected

B. Cycle number versus Delta Rn from a representative experiment of Taqman real-time PCR amplification genomic DNA isolated from HeLa cells 48 hrs after infection with AAV-Rep-GFP vector, AAV-GFP vector or Mock infected.

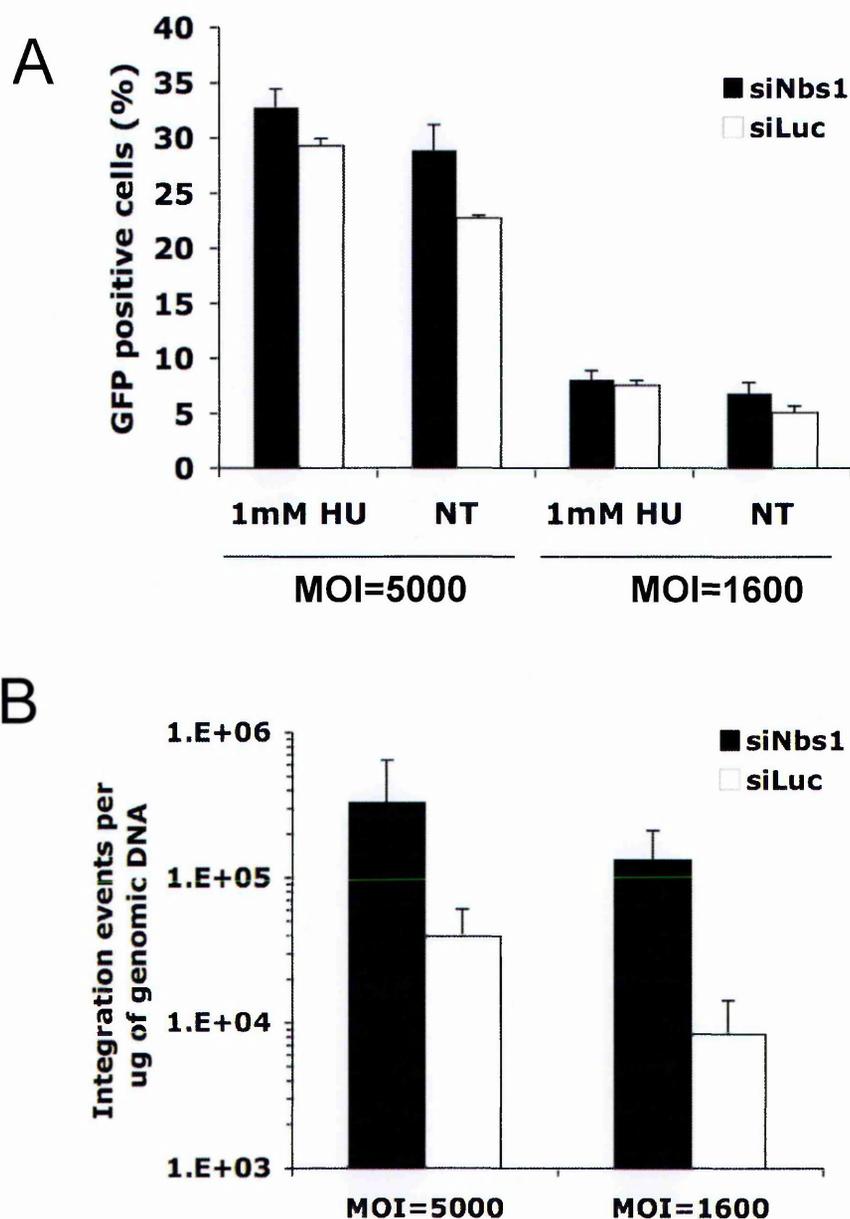


Figure 6.13. Effect of Nbs1 silencing on AAV-Rep-GFP transduction and site-specific integration.

A. Flow cytometry analysis of HeLa cell after transduction with AAV-GFP. Cells were transfected with siRNA against Nbs1 (siNbs1) or with a control siRNA against Luciferase (siLuc) and either with or without hydroxyurea (HU).

B. Quantification of Rep mediated site-specific integration. Cells were transfected with siRNA against Nbs1 (siNbs1) or with a control siRNA against Luciferase (siLuc), 48 hrs before infection with AAV-Rep-GFP. Number of integration events per μg of genomic DNA is expressed in logarithmic scale.

Transduction and site-specific integration experiments were conducted at MOI of 1,600 and 5,000 vgp/cell.

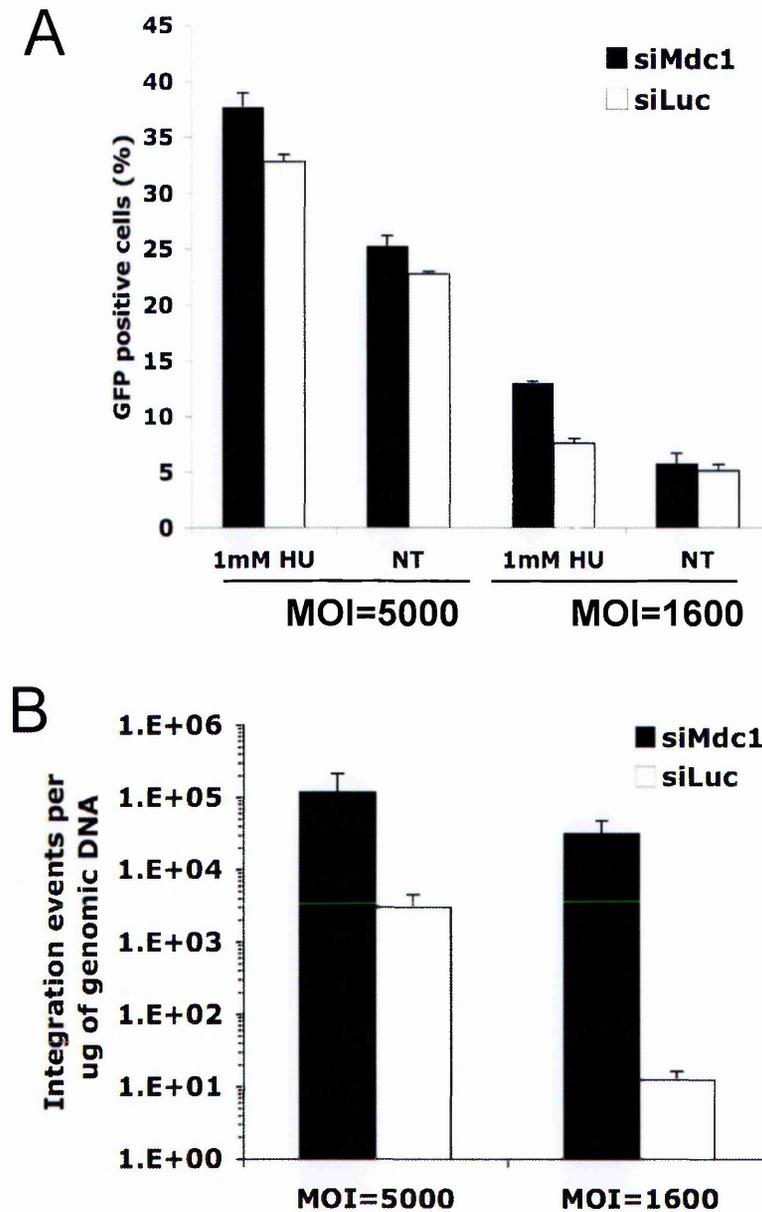


Figure 6.14. Effect of Mdc1 silencing on AAV-Rep-GFP transduction and site-specific integration.

A. Flow cytometry analysis of HeLa cell after transduction with AAV-GFP. Cells were transfected with siRNA against Mdc1 (siMdc1) or with a control siRNA against Luciferase (siLuc) and either with or without hydroxyurea (HU).

B. Quantification of Rep mediated site-specific integration. Cells were transfected with siRNA against Mdc1 (siMdc1) or with a control siRNA against Luciferase (siLuc), 48 hrs before infection with AAV-Rep-GFP. Number of integration events per μg of genomic DNA is expressed in logarithmic scale.

Transduction and site-specific integration experiments were conducted at MOI of 1,600 and 5,000 vgp/cell.

6.2.4. Silencing of Rad52 decreases the frequency of Rep dependent site-specific integration.

We have shown that silencing of Rad52 protein resulted in significant decrease in cell permissivity to rAAV transduction (Figure 6.10A); this trend was also reproduced for the AAV-Rep-GFP vector (from 2.1 to 2.3 fold decrease in the percentage of GFP positive cells with HU pre-treatment, and from 1.7 to 1.9 fold decrease without HU treatment), at MOIs of 5,000 and 1,600 vgp/cell (Figure 6.15A).

The striking observation was that we were not able to detect any integration of the AAV vector by real-time PCR technique at the MOIs of 5,000 and 1,600 vgp/cell in samples in which the Rad52 protein was silenced (Figure 6.15B). A similar reduction in integration was observed when cells were transfected with a pSuper plasmid coding for a hairpin siRNA against Rad52 (pSuper-Rad52) (Figure 6.15C). Previous chromatin immunoprecipitation experiments conducted in our laboratory indicating that Rad52 protein interacts physically with rAAV genomes (Zentilin et al., 2001) prompted us to hypothesize that Rad52 may be directly required during AAV site-specific integration.

6.2.5. Silencing of DNA-PKcs decreases frequency of Rep-dependent site-specific integration.

Similar to the results obtained after evaluating the effect of Rad52 silencing in the presence and in the absence of Rep protein, transduction of rAAV-Rep-GFP vector was significantly reduced after siRNA-mediated silencing of DNA-PKcs in HeLa cells (Figure 6.16A). This reduction was observed in cells both treated or not with HU at MOI=5,000 (from $32.8 \pm 0.7\%$ to $19.5 \pm 9.3\%$ with HU treatment, and from $22.8 \pm 0.2\%$ to $15.3 \pm 0.8\%$ without HU treatment).

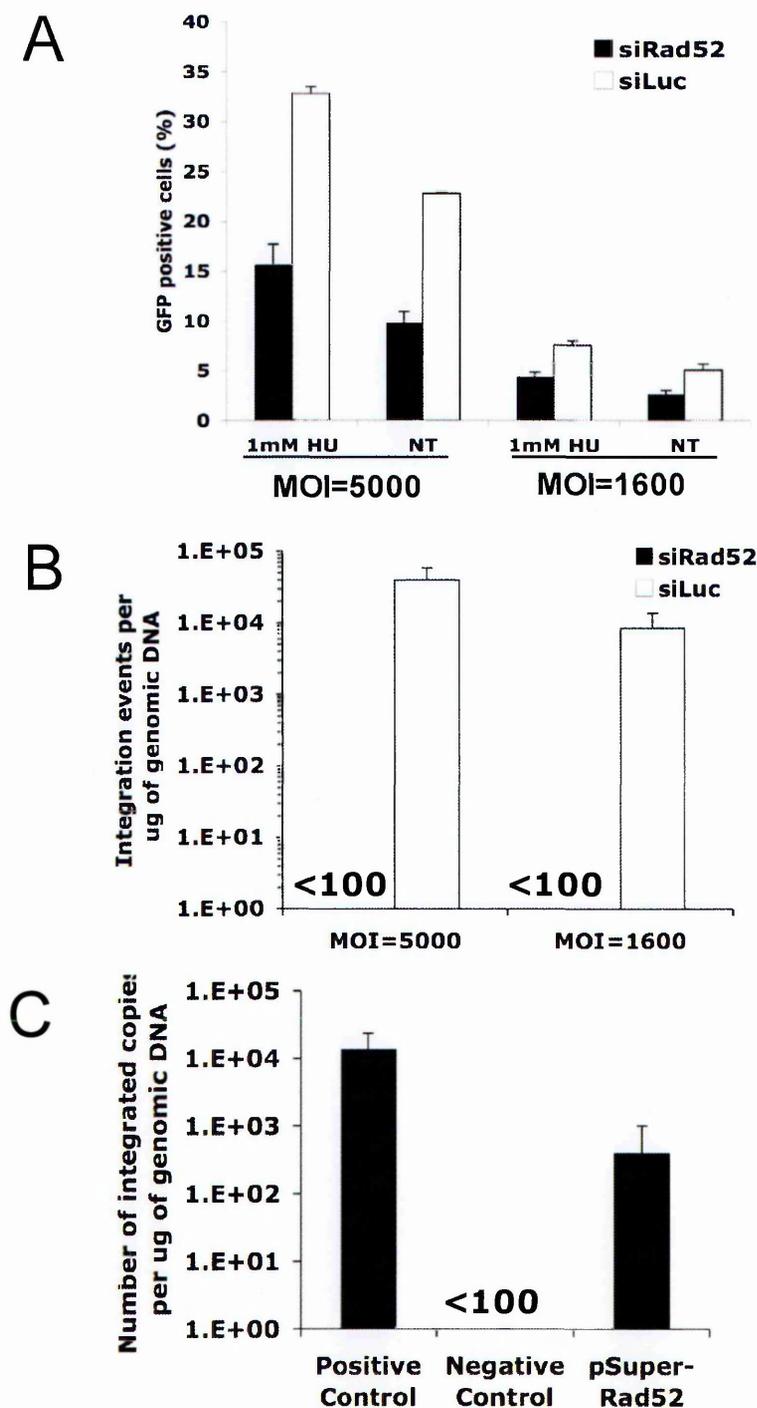


Figure 6.15. Effect of Rad52 silencing on AAV-Rep-GFP transduction and site-specific integration.

A. Flow cytometry analysis of HeLa cell after transduction with AAV-GFP. Cells were transfected with siRNA against Rad52 (siRad52) or with a control siRNA against Luciferase (siLuc) and either with or without hydroxyurea (HU). Infections were conducted at MOIs of 1,600 and 5,000 vgp/cell.

B. Quantification of Rep mediated site-specific integration. Cells were transfected with siRNA against Rad52 (siRad52) or with a control siRNA against Luciferase (siLuc), 48 hrs before infection with AAV-Rep-GFP. Number of integration events per μg of genomic DNA is expressed in logarithmic scale. <100 stands for Non-detectable. Infections were conducted at MOIs of 1,600 and 5,000 vgp/cell.

C. Quantification of Rep mediated site-specific integration. Cells were transfected with pSuper-Rad52 or with a control pSuper-GFP, 48 hrs before infection with AAV-Rep-GFP. Number of integration events per μg of genomic DNA is expressed in logarithmic scale. Infection was conducted at a MOI of 1,600 vgp/cell.

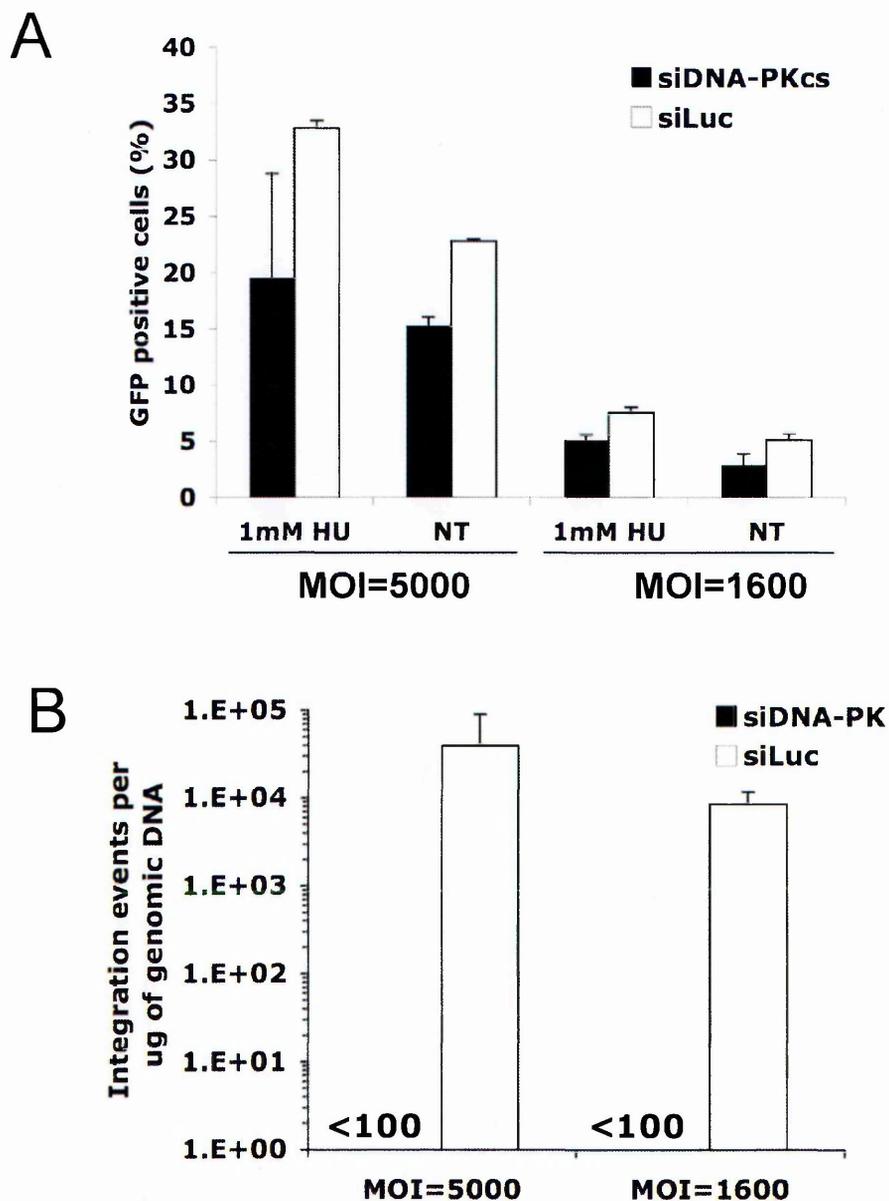


Figure 6.16. Effect of DNA-PKcs silencing on AAV-Rep-GFP transduction and site-specific integration.

A. Flow cytometry analysis of HeLa cell after transduction with AAV-GFP. Cells were transfected with siRNA against DNA-PKcs (siDNA-PKcs) or with a control siRNA against Luciferase (siLuc) and either with or without hydroxyurea (HU).

B. Quantification of Rep mediated site-specific integration. Cells were transfected with siRNA against DNA-PKcs (siDNA-PKcs) or with a control siRNA against Luciferase (siLuc), 48 hrs before infection with AAV-Rep-GFP. Number of integration events per μg of genomic DNA is expressed in logarithmic scale. <100 stands for Non-detectable.

Transduction and site-specific integration experiments were conducted at MOI of 1,600 and 5,000 vgp/cell.

Previous studies have shown that DNA-PKcs may play an inhibitory role at the level of site-specific integration (Song et al., 2004). In disagreement with these findings, our results show that the siRNA-mediated silencing of DNA-PKcs produced a decrease in site-specific integration (Figure 6.16A). This discrepancy could be due the fact that Song and collaborators conducted their experiments using an *in vitro* integration assay, while additional levels of complexity might well be present in living cells.

6.2.6. Silencing of H2AX decreases frequency of Rep-dependent site-specific integration.

Parallel to what has been previously described for Rad52 and DNA-PKcs, silencing of the H2AX histone decreased the permissivity of the cells to AAV-Rep-GFP transduction, both in the presence and absence of HU treatment at a MOI of 5,000 vgp/cell (Figure 6.17A). At lower MOIs a difference in cells permissivity was not observed.

Site-specific integration at the AAVS1 locus was also reduced to the point of becoming undetectable, both in the cells infected with a MOI of 5,000 as well at a MOI of 1,600 (Figure 6.17B).

6.3. Cell cycle profile after silencing of Nbs1, Mdc1, H2AX, Rad52 and DNA-PKcs.

The knocking down of the proteins that are the subject of this study could affect the normal progression of the cell cycle. Since there is still some controversy about the dependence of rAAV transduction efficiency in cell culture on the cell cycle (Alexander et al., 1994a; Alexander et al., 1996; Russell et al., 1995; Russell et al., 1994; Yakobson et al., 1987), we analyzed the possible

association between a specific cell cycle profile and the differences in transduction of rAAV observed after silencing. There are no studies indicating whether AAV site-specific integration may be favored during any particular phase of the cell cycle, however, AAV mediated gene targeting which depends on the HR machinery is favored, as expected, during S-phase (Trobridge et al., 2005).

To rule out the possibility that the differences in transduction efficiency that we observe are due to cell cycle arrest after RNAi silencing, we transfected HeLa cells with siRNAs against Nsb1, Mdc1, H2AX, DNA-PKcs, and Rad52 and determined their cell cycle profile by FACS analysis at the moment of maximum silencing. The results shown in Figure 6.18 indicate that only very minor changes were observed after RNA silencing of any of the investigated proteins; no correlation could be detected between the observed cell cycle profiles and the extent of rAAV transduction. Cells treated with HU, irrespectively of the siRNA used, showed cell cycle arrest in G1/S, as expected.

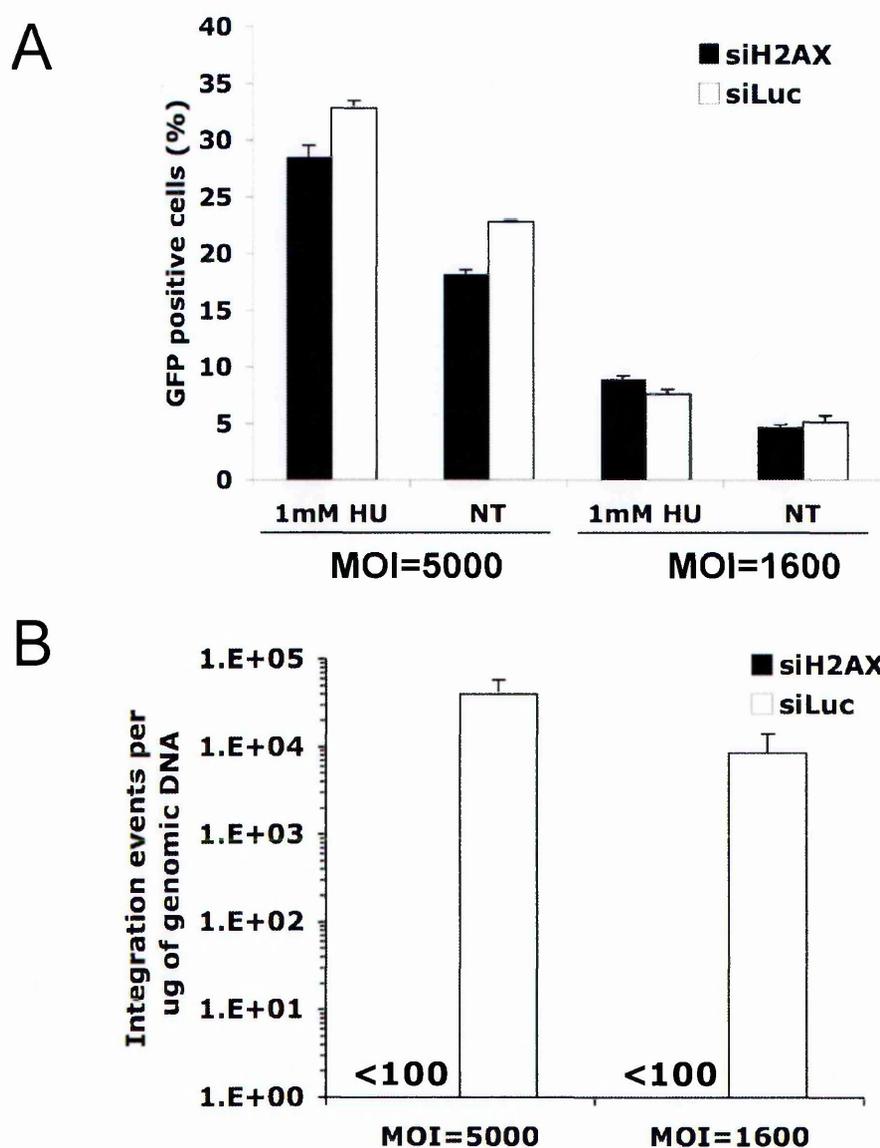


Figure 6.17. Effect of H2AX silencing on AAV -Rep-GFP transduction and site-specific integration.

A. Flow cytometry analysis of HeLa cell after transduction with AAV-GFP. Cells were transfected with siRNA against H2AX (siH2AX) or with a control siRNA against Luciferase (siLuc) and either with or without hydroxyurea (HU).

B. Quantification of Rep mediated site-specific integration. Cells were transfected with siRNA against DNA-PKcs (siDNA-PKcs) or with a control siRNA against Luciferase (siLuc), 48 hrs before infection with AAV-Rep-GFP. Number of integration events per μg of genomic DNA is expressed in logarithmic scale. <math><100</math> stands for Non-detectable.

Transduction and site-specific integration experiments were conducted at MOI of 1,600 and 5,000 vgp/cell.

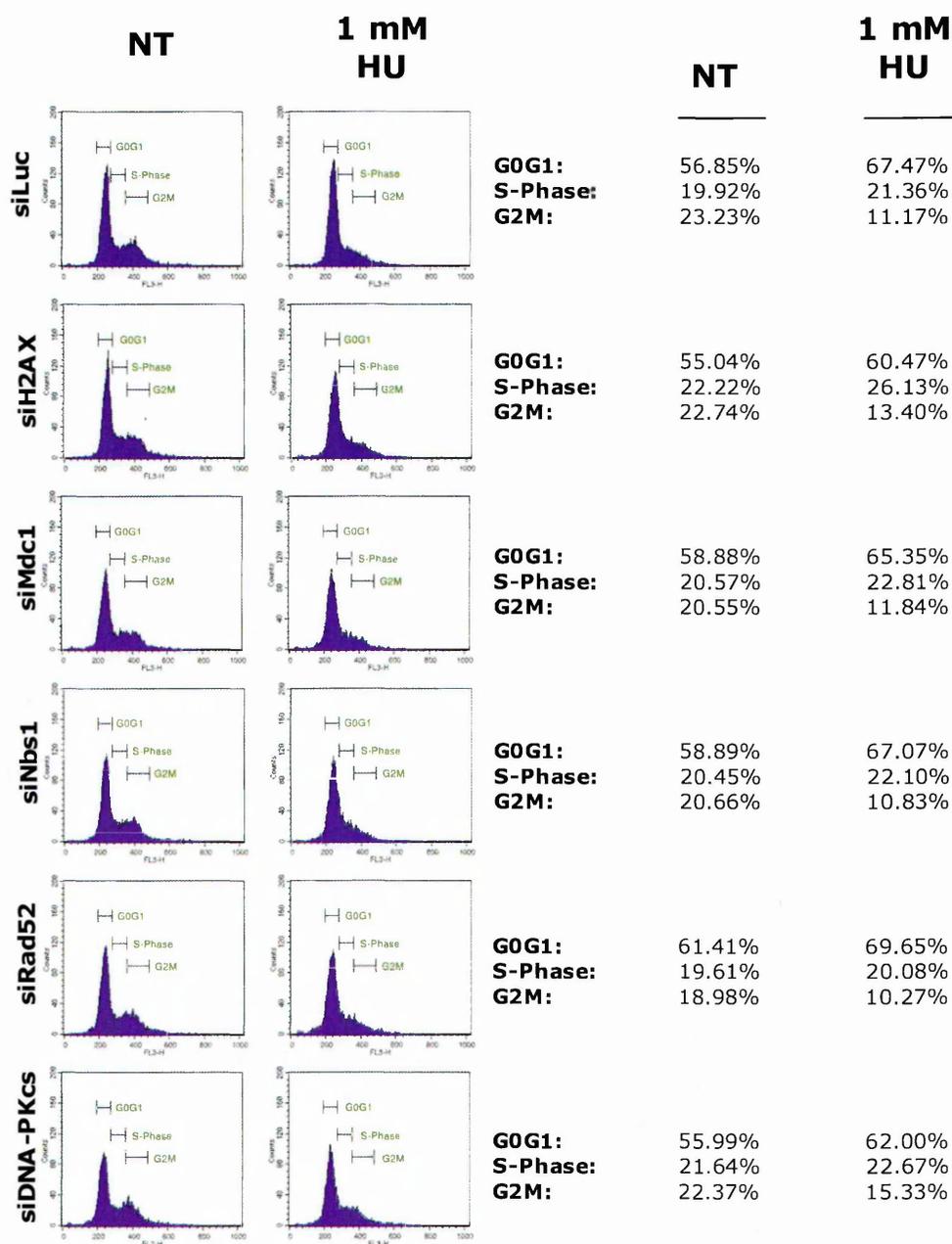


Figure 6.18. Cell cycle profile after siRNA mediated silencing against H2AX, Mdc1, Nsb1, Rad52 and DNA-PKcs.

Cell cycle profile of HeLa cells after transfection with siRNA against Luciferase (siLuc), H2AX (siH2AX), Mdc1 (siMdc1), Nbs1 (siNbs1), Rad52 (siRad52), DNA-PKcs (siDNA-PKcs), with or without hydroxyurea treatment (HU and NT).

7. DISCUSSION

7. 1. DNA Repair machinery and its influence on rAAV transduction and genome processing:

Despite the valuable properties of rAAV vectors for *in vivo* gene transfer, the molecular correlates of rAAV transduction are still poorly understood. Due to the single-stranded nature of the AAV genome, its processing into a double-stranded, transcriptionally active molecular species clearly represents a fundamental event. This notion is nourished by *in vivo* studies that showed that long-term transgene expression mediated by rAAV vector correlates with the formation of double-stranded, circular and concatamerized vector genomes (Duan et al., 1998; Schnepf et al., 2005; Yang et al., 1999).

In this study, we have exploited live imaging microscopy to assess the kinetics of double-stranded rAAV DNA accumulation in the nucleus of transduced cells, in the absence of viral replication or expression of any helper virus gene product. The developed system takes advantage of the interaction of a EGFP-LacR fusion protein, expressed at low levels in the nucleus of the transduced cells, with 112 repeats of the cognate LacO sequence cloned into a rAAV vector. Since LacR specifically binds dsDNA, this approach permits the visualization of rAAV genomes once converted from ssDNA to dsDNA inside the nucleus. By using this approach in different cell types, we observed that rAAV dsDNA accumulation does not occur diffusely in the nucleoplasm, but is confined to specific rAAV foci, which become detectable as early as 3 h after cell infection and progressively increase in size and intensity of fluorescence over the first 12 h after infection, suggestive of a progressive accumulation of viral dsDNA genomes into these structures. A detailed biophysical analysis of rAAV foci by time lapse imaging studies indicates that these nuclear structures are rather stable over time

and are relatively immobile. Moreover, these fluorescent foci do not appear associated with the insoluble chromatin compartment of the nucleus, since they completely disappear when AAV-LacO.14 transduced cells are treated with mild detergents before fixation (data not shown). Given the sensitivity threshold of conventional fluorescence microscopy, as well as the fluorescent behavior of single EGFP molecules (Cinelli et al., 2000), it is clear that each of the rAAV foci comprises several dsDNA molecules, which might well correspond to multimerized rAAV genomes, as shown by a number of studies that analyzed the molecular nature of transcription-competent AAV genomes in the transduced cells (Afione et al., 1996; Flotte, 2004; Yang et al., 1999).

Cell treatment with HU is known to significantly boost the efficiency of rAAV transduction (Russell et al., 1995; Zentilin et al., 2001). In our experiments, we observed that the same treatment also increased the number of cells with rAAV foci, thus showing that the formation of rAAV foci positively correlates with the transgene transduction. Both in the presence or absence of HU treatment, the number of cells with rAAV foci peaked between 24 and 48 h post infection, and then gradually decreased. This decrease may be well due to the slow degradation of some rAAV genomes or to their migration out of the foci to the nucleoplasm.

Previous studies with wt AAV in Adenovirus-infected cells have shown, by immunofluorescence and *in situ* hybridization, that, during infection, wt AAV DNA replication occurs in discrete nuclear compartments (Weitzman et al., 1996; Wistuba et al., 1997). Similar findings have been also confirmed in live cells infected with HSV-1 or transfected with HSV-1 DNA, by using a rAAV vector containing only 40 tandem repeats of a LacO binding sites and a reporter protein similar to ours (Fraefel et al., 2004). The presence of these AAV foci has been

interpreted as an indication of the existence of nuclear compartments in which AAV DNA replication takes place. The observation that both the Rep proteins and helper virus DNA also co-localized with these foci is fully consistent with this interpretation (Fraefel et al., 2004; Glauser et al., 2005; Weitzman et al., 1996). The rAAV foci we detect in our experiments might well be coincident with the nuclear compartments where AAV and Adenovirus replication occurs. However, it should be pointed out that the rAAV foci we observe are not determined by the presence of any expressed viral protein normally involved in replication. Instead, our experiments indicate the existence of specific nuclear compartments that have strong affinity for rAAV DNA in the absence of viral or helper proteins, and thus suggest that rAAV DNA is specifically recognized by cellular factors.

Indeed, we found that rAAV foci partially colocalize or are closely juxtaposed to the nuclear compartments where MRN complex proteins accumulate upon treatment of the transduced cells with HU or other DNA damaging agents (UV and camptothecin, data not shown). In support of the notion that these proteins are actively involved in a DNA repair process, we observed that rAAV foci associated with foci of a DNA damage-activated form of Nbs1, phosphorylated at serine 343, (Gatei et al., 2000; Lim et al., 2000; Wu et al., 2000). MRN complex is known to bind both ss and dsDNA and to possess a pivotal role in sensing damaged or hairpin-structured DNA (D'Amours and Jackson, 2002; Petrini and Stracker, 2003; van den Bosch et al., 2003). In keeping with the possibility that the DNA double strand break repair machinery is involved in rAAV genome processing, we found that an upstream mediator, Mdc1, known to increase the retention of Nbs1 at the sites of DNA damage (Lukas et al., 2004), also formed foci that partially colocalized with the rAAV foci.

What might be the role of the MRN proteins and of Mdc1 in rAAV genome processing? Our RNAi experiments clearly suggest that the overall effect of these proteins is inhibitory in rAAV dsDNA formation. Silencing of Nbs1 by RNAi increased the number of cells containing rAAV foci and significantly improved the permissivity of both HeLa and MRC5 cells to transduction, again indicating that generation of dsDNA, and thus formation of rAAV foci, positively correlates with cell permissivity to transduction. Similar considerations also apply to the silencing of Mdc1. Interestingly, Nbs1 silencing had a marked effect in increasing the number of cells with AAV foci; even if it modified only modestly the average number of foci that were present per cell.

Over the last few years, we and others have reported that cells lacking functional ATM are markedly more permissive to rAAV transduction (Sanlioglu et al., 2000; Zentilin et al., 2001). The observation that Nbs1 silencing has no apparent effect on the level of transduction in ATM-defective cells is consistent with the possibility that the ATM protein might mediate the negative regulation of MRN protein activity on the incoming rAAV genomes. In this respect, it is worth noting that ATM and Nbs1 have been recently shown to be essential in the formation of the replication protein A-coated ssDNA microcompartments following cell irradiation with ionizing radiations (Bekker-Jensen et al., 2006). It might thus be envisioned that these proteins also participate in the processing of the ssDNA AAV genomes. It is worth noting that the increase in transduction efficiency after Nbs1 silencing was observed both in the absence and presence of HU. Indeed, the major effect of the HU treatment is to enhance the outcome of Nbs1 silencing on AAV cell transduction. This suggests that the formation of MRN foci is not a pre-requisite for the accumulation of rAAV into nuclear foci

and that they are not necessarily associated with the positive effect on AAV transduction observed upon Nbs1 silencing.

Productive AAV replication requires coinfection with unrelated helper virus, such as Adenovirus or transfection of its helper genes. It is known that adenovirus E1B55K and E4orf6 proteins enhance rAAV transduction and wt AAV replication, by facilitating the conversion of the single stranded to second stranded viral genome synthesis through a mechanism that is not yet fully described (Ferrari et al., 1996; Fisher et al., 1996). E1b55K and E4orf6 form a complex that has been recently shown to possess ubiquitin ligase activity in conjunction with cellular proteins, and to promote degradation of host targets p53 and the MRN complex (Harada et al., 2002; Querido et al., 2001; Stracker et al., 2002). Adenovirus proteins promote MRN degradation to prevent DNA damage signaling and viral genome processing (Carson et al., 2003; Stracker et al., 2002).

Here we have shown that AAV is a target of MRN, and, therefore, the degradation of these proteins by E1b55K/E4orf6 creates a more permissive cellular environment for AAV replication and transduction (Figure 6.7). Consequently, it is conceivable to suppose that AAV may have evolved to rely on Adenovirus functions in order to get rid of the DNA repair proteins that hinder its infection process. In the present work, in collaboration with M.D. Weitzman (Salk Institute, La Jolla), we have also shown that the degradation of MRN by the Adenovirus proteins E4orf6/E1B55k results in an increase in rAAV transduction efficiency. Moreover, we have shown that this effect occurs independent of the degradation of p53 (Schwartz et al, 2006, manuscript under revision, EMBO Reports).

As expected, E4orf6/E1B55k-mediated degradation of MRN also increases the number of cells with rAAV foci. These findings, together with the

results obtained after siRNA mediated silencing of Nbs1, further confirm that the MRN complex plays an inhibitory role at the level of rAAV genome processing and cell transduction.

Recent studies have shown that wild-type and recombinant adeno-associated virus (AAV and rAAV) genomes persist in human tissue predominantly as double-stranded (ds) circular episomes derived from input linear single-stranded virion DNA (Duan et al., 2003; Song et al., 2001). These data strongly suggest that wt and recombinant AAV genomes utilize similar host recombination pathways. While the correlation between long-term persistence of rAAV genomes and conversion to circular and concatameric forms has been noted in numerous studies, there is no clear consensus about the fate of the linear rAAV genomes. These molecular species are likely to represent a transient episomal phase due to the recombinogenic nature of the free DNA ends. It is therefore important to understand how the mammalian DNA repair and recombination machinery participate in the conversion of linear rAAV genomes to more stable structures. Undoubtedly, persistence of the vector genome in the host cell is a critical parameter for successful use of rAAV for gene delivery.

The homologous recombination (HR) as well as non-homologous end joining (NHEJ) DNA repair machineries have been implicated in different steps of rAAV transduction (Choi et al., 2006; Jurvansuu et al., 2005; Song et al., 2001; Song et al., 2004; Vasileva et al., 2006; Zentilin et al., 2001).

Previous experiments conducted in our laboratory have shown that, direct interaction of Rad52 with rAAV genomes, evaluated by quantitative chromatin immunoprecipitation, correlates with higher transduction efficiency (Zentilin et al., 2001). As expected, siRNA mediated silencing of Rad52 resulted in a decrease in transduction efficiency (Figure 6.10A).

On the other hand, several previous studies have demonstrated that NHEJ repair proteins associate with the rAAV genomes and affect their molecular fate in different ways. It has been shown that the catalytic subunit of DNA-PK (DNA-PKcs) affects the efficiency of rAAV genome circularization. In mice lacking DNA-PKcs (SCID mice), rAAV vector DNA recovered from muscle contains a significant fraction of linear molecules, which are not seen in normal mice (Duan et al., 1998; Song et al., 2001). Our experiments show a dependence of rAAV (Rep-) transduction on DNA-PKcs only when cells are treated with HU, suggesting that the requirements of this factor would be essential in a particular phase of the cell cycle (i.e. G1/S) or in the presence of cellular DNA damage response. Further characterization in this respect will be necessary.

Phosphorylation of histone H2AX at serine 139 (γ -H2AX) is one of the first events after DNA damage response (Riballo et al., 2004; Rogakou et al., 1998). Previous studies have suggested that the cell surveillance machinery may recognize AAV genomes as stalled replication forks (Jurvansuu et al., 2005). The well recognized involvement of H2AX phosphorylation in DNA repair after collapse of the replication fork (Furuta et al., 2003; Mirzoeva and Petrini, 2003) makes us think about the possible participation of H2AX in the rAAV genome processing. Interestingly, silencing of H2AX produces a decrease in rAAV transduction in the presence and absence of HU treatment; however, when DNA damage is induced by HU, the reduction on rAAV transduction was more pronounced (Figure 6.10C). Nevertheless, it is important to mention that it is not known what might be the chromatin structure of rAAV genomes once inside the cell nucleus.

7. 2. DNA Repair machinery and AAV site-specific integration.

One of the most intriguing aspects of AAV biology is represented by the unique ability of this virus to integrate site-specifically into the human genome, at Ch19 (Berns and Linden, 1995; Linden and Berns, 2000). Several strategies have been used to evaluate the efficiency of AAV site-specific integration into Ch19. Initial estimates of the efficiency of this process were based on the number of rescue-competent, latent infections that could be derived from a pool of infected cells (McCarty et al., 2004). In these studies, the AAV integration efficiencies achieved in infected cells were typically of 20%–80%. Similar results have been obtained using Rep-containing vectors (McLaughlin et al., 1988; Mendelson et al., 1988; Samulski et al., 1989). More recently, Huser *et al.* (2002) have developed a rapid and efficient assay for Ch19 specific integration, based on polymerase chain reaction (PCR) amplification using primers specific for the AAV and flanking chromosomal sequences. Using this technique, they reported values of integration frequency that are consistent with earlier results and with the frequencies we have observed in our experiments. The use of quantitative PCR (qPCR) to quantify the number of site-specific integration has several advantages as well as some limitations that are inherent to the method and the nature of the AAV integration process. The technique used by us as well as the one described by Huser *et al.* (2002) have the advantage to be highly sensitive, fast and easily adaptable to high throughput applications. It allows us to detect and quantify the number of integration events in the absence of selective pressure against the cell that harbor the integrated viral genomes, and to collect the samples at any time point during the experiment without the requirement of enrichment of any cell population. However, the exact number of integration events needs to be analyzed carefully. It is important to mention that even if the Rep-mediated

integration of AAV genome occurs at high frequency along the AAVS1 locus, the break point is not identifiable and fixed. As a consequence, every single integration event may be potentially different from cell to cell, and eventually the length of the fragments produced after PCR amplification is variable (Huser et al., 2002). Therefore, the possibility always exists that shorter PCR amplified fragments may be favored during the amplification reaction, resulting in a possible underestimation of the totality of the integration events in a given sample. Even in the light of these considerations, this experimental approach is considered the most flexible and convenient for the kind of experiments conducted in this study and many other biotechnological applications (McCarty et al., 2004).

It is known that the Rep protein is necessary for AAV site-specific integration into Ch19, but the reason why this event takes place selectively at the AAVS1 locus, the mechanism of integration, and the role of cellular factor in this process remain elusive (Hamilton et al., 2004; Vasileva and Jessberger, 2005). In this study we attempt to identify cellular factors that may participate directly or indirectly in the process of Rep-dependent AAV site-specific integration. The remarkable inhibitory role of the proteins of the MRN complex at the level of rAAV genome processing and transduction prompted us to explore its possible participation also in the process of genome integration. Interestingly, silencing of Nbs1 as well as Mdc1 dramatically increased the frequency of integration events, suggesting that these proteins may interfere with the processing of the AAV genomes required for chromosomal integration. This line of thought is favored by the observation that the transduction efficiency of the AAV-Rep-GFP vector, after silencing of these proteins, was not dramatically affected (Figures 6.13 and 6.14).

One possible explanation for the above findings could take into account

the known property of the Rep protein to induce DNA breaks into the host cell genome. The MRN complex would be eventually recruited at these sites and possibly titrated out from the AAV genomes (Berthet et al., 2005). Alternatively, it is tempting to speculate that the Rep proteins would establish interactions with proteins involved in the repair of double strand breaks and acting as sensors, effectors or signal transducers and modulators of the cell cycle. In this respect, two examples of such interactions are the reported binding between Rep68 and Topors, a p53 and topoisomerase I binding protein (Weger et al., 2002), and between Rep68 and Replication protein A (RPA) (Stracker et al., 2004).

On the other hand, H2AX, Rad52 and DNA-PKcs silencing have shown to markedly decrease, in our experimental conditions, the integration efficiency. In the case of Rad52, a decrease in site-specific integration was also accompanied by a reduction in AAV-Rep-GFP transduction efficiency, measured as the percentage of cells positive for EGFP. Recent publications have indicated that the HR machinery participates in the process of AAV-mediated gene targeting, a process that could be, from a mechanistically point of view, similar to the Rep dependent site-specific integration (Vasileva et al., 2006). As previously discussed, Rad52 proteins are known to physically interact *in vivo* with rAAV vector genomes (Zentilin et al., 2001). In light of these results, the Rad52 protein may directly participate in the process of integration or may be necessary for a molecular modification prior the integration event, such as strand annealing between AAV genomes of positive and negative polarities or annealing with homologous genomic regions.

It has been observed, however, that no large regions of homology are found between the AAVS1 and the wtAAV, and it has been thus suggested that integration occurs through a non-homologous end-joining pathway (Russell,

2003). Small (4–5-bp) homologies at the junctions between host cell and viral DNA are consistent with illegitimate recombination products. Partial deletion of sequences within the AAV ITRs, as well as large-scale rearrangements of the host sequences around the integration site, suggested that the process is both complex and imprecise (Hamilton et al., 2004).

Transduction and site-specific integration efficiency of the Rep-containing AAV vector were significantly reduced after siRNA mediated silencing of both Rad52 and DNA-PKcs, suggesting a very complex interplay between different cellular factors. As already discussed (See section D.1), transduction efficiency of AAV-LacZ (Rep-) showed only dependence on DNA-PKcs after treating the cells with HU; this observation suggests a potentially differential usage, by AAV, of the cell machinery depending on the cell environment and its position in the cell cycle.

γ -H2AX foci formation has been observed in cells expressing wt AAV Rep protein (Berthet et al., 2005). Since the insertion of the AAV genome into the AAVS1 locus would require the cleavage of the chromosomal DNA, probably mediated by the Rep protein (Hamilton et al., 2004), in the absence of H2AX, the recruitment of the DNA repair machinery required for sealing the broken ends created during, may be impaired.

This could explain our observed decrease in site-specific integration after H2AX silencing. However, further studies would be necessary to clarify the actual role of H2AX in this process.

7.3. Final considerations

On the basis of the results presented in this thesis, we would like to propose a model that explains the fate of rAAV DNA once inside the host cell nucleus, which entirely depends on the host cell machinery (Figure 7.1). According to this model, rAAV genomes, by virtue of both their ssDNA nature and the presence of secondary DNA structures in the viral ITRs, are recognized by cellular proteins involved in the DNA damage response; including MRN, Mdc1 and components of the HR and NHEJ machinery. MRN complex proteins and Mdc1 are inhibitory of ssDNA to dsDNA genome conversion, and, thus, are detrimental for efficient transduction. Only once this inhibition is eluded, the genomes might be converted to dsDNA by either second strand DNA synthesis utilizing the hairpin ITRs in a self-priming replication mechanism, or by the direct annealing of the complementary DNA strands of the incoming vectors (Fisher et al., 1996; Hauck et al., 2004). Cell treatment with DNA damaging agents might divert the inhibitory proteins away from the rAAV genomes, and this would explain the positive effects of genotoxic agents on rAAV transduction. Consequently, degradation of MRN complex by means of Adenovirus E1B55k/E4orf6 complex exerts a positive effect on AAV transduction similar to the one observed after HU treatment.

After ssDNA to dsDNA conversion, the vector genome is known to undergo additional changes, which are mediated by host cell factors acting on the AAV ITR ends. These changes involve circularization of the vector DNA, or the formation of end-to-end concatamers (reviewed in McCarty et al., 2004). Cellular proteins that participate in HR and NHEJ may either play a role at this level or, more likely as in the case of Rad52, in the process of annealing of rAAV genomes of positive and negative polarity.

It is possible to speculate that histone H2AX may get phosphorylated at the moment of concatamer formation and/or at the moment of integration.

H2AX may be assembled into the AAV dsDNA genomes, as well as present in the target genomic AAVS1 locus. Phosphorylation of either viral or AAVS1 H2AX may be required to trigger a DNA damage response that will end with the integration of the viral DNA into the host chromosome. Further studies about the structure and composition of the rAAV chromatin may help to explain these findings.

A recent work that exploited self-complementary AAV vectors, which bypass the ssDNA to dsDNA conversion step, because their genomes are already dsDNA, has shown that ATM and MRN proteins participate in AAV genome circularization (Choi et al., 2006). Thus, it might be envisioned that these proteins, while being inhibitory on the input single-stranded viral genome either by impeding dsDNA synthesis or by routing the genomes to aberrant processing or nucleolytic degradation, might become positive factors when dsDNA synthesis has already occurred. At this stage, resolution of the secondary structures in the ITRs by promoting circulation or multimerization might be essential to allow stable maintenance of the viral genomes inside the nucleus.

Does genome integration require the formation of dsDNA AAV genomes? The clarification of the exact nature of the substrate required for site-specific integration is of fundamental importance; in this respect the use of self-complementary Rep containing AAV could give some valuable information.

H2AX, Rad52 and DNA-PKcs proteins may be required for the processing of the AAV in order to generate multimerized genomes competent for transcription or/and suitable substrate for Rep dependent site-specific integration.

On the other hand, the proteins of the MRN complex and Mdc1 exert inhibitory roles both at transcription and at site-specific integration.

Finally, we wish to emphasize that one of the most challenging but still unanswered questions related to rAAV vectors is the explanation, in molecular terms, of their exquisite efficiency in post-mitotic tissues *in vivo*, such as brain, retina and heart (Carter et al., 2004). Our findings prompt the investigation of the levels and activity of MRN complex proteins, HR and HHEJ pathways in these tissues.

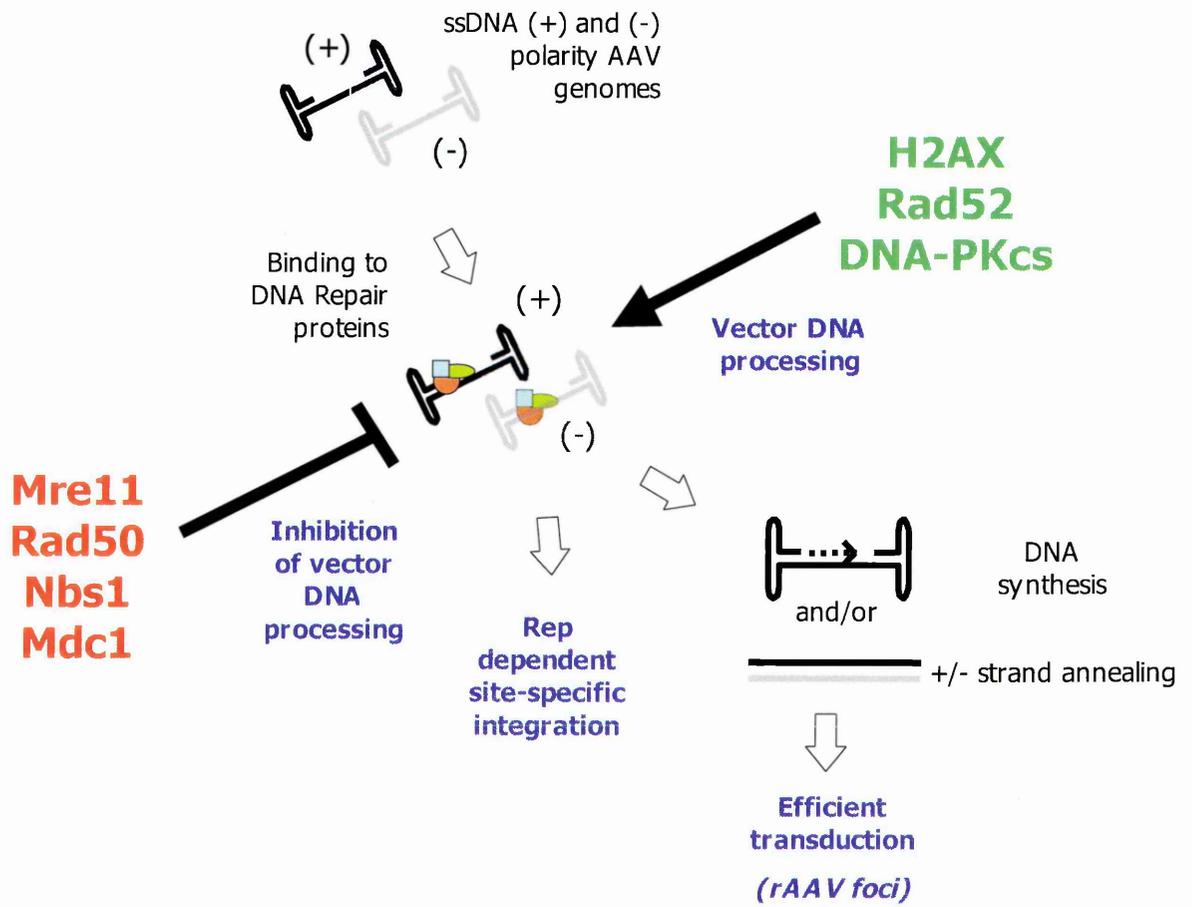


Figure 7.1. Interaction of AAV with the DNA DSB repair machinery

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