Scuola Internazionale Superiore di Studi Avanzati - Trieste

AMF-1/Gps2 and ANP32B are novel cellular co-factors that modulate Adeno-Associated virus type 2 Rep activity

Gianluca Pegoraro

Thesis submitted for the degree of

Doctor Philosophiae

Supervisors:
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Academic Year 2003/2004

SISSA – Via Beirut 2-4 – 34014 TRIESTE – ITALY

ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

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Ad Augusto ed Elisabetta

Index

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: Results

Chapter 1: Introduction.

1.1 The Adeno Associated Virus Type2 (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 can establish a latent infection in the host cell by integrating site specifically on the long arm of human chromosome 19 (19q13-qter) (Kotin et al., 1992; Kotin et al., 1991; Kotin et al., 1990; Samulski et al., 1991). Once integrated, the provirus can be 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).

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 *Paroviridae* family, genus *Dependovirus* (Siegl et al., 1985). Furthermore, more recently it was possible to isolate different AAVs based on their serological properties (From AAV1 to AAV-8, for a review see (Grimm and Kay, 2003)). 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 correlation pathogenic role for AAV, it has been impossible to correlate infection with any known disease, despite 90% of the American population is seropositive (Blacklow et al., 1971). More recently, some reports showed the presence of AAV DNA in 40% of the samples obtained from

spontaneous abortions, but drawing definitive conclusions from this data seems highly premature (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, 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. Thus, nowadays, new discoveries on AAV are the result of an active interplay between basic and applied science.

1.2 The AAV-2 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 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 Fig. 1.1).

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 Fig 1.2). To maximize base pairing and thermodinamical 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 tetra-repeat of the GAGC sequence (Chiorini et al., 1994a; Chiorini et al., 1994b; Owens et al., 1993); the RBS', in one of the two

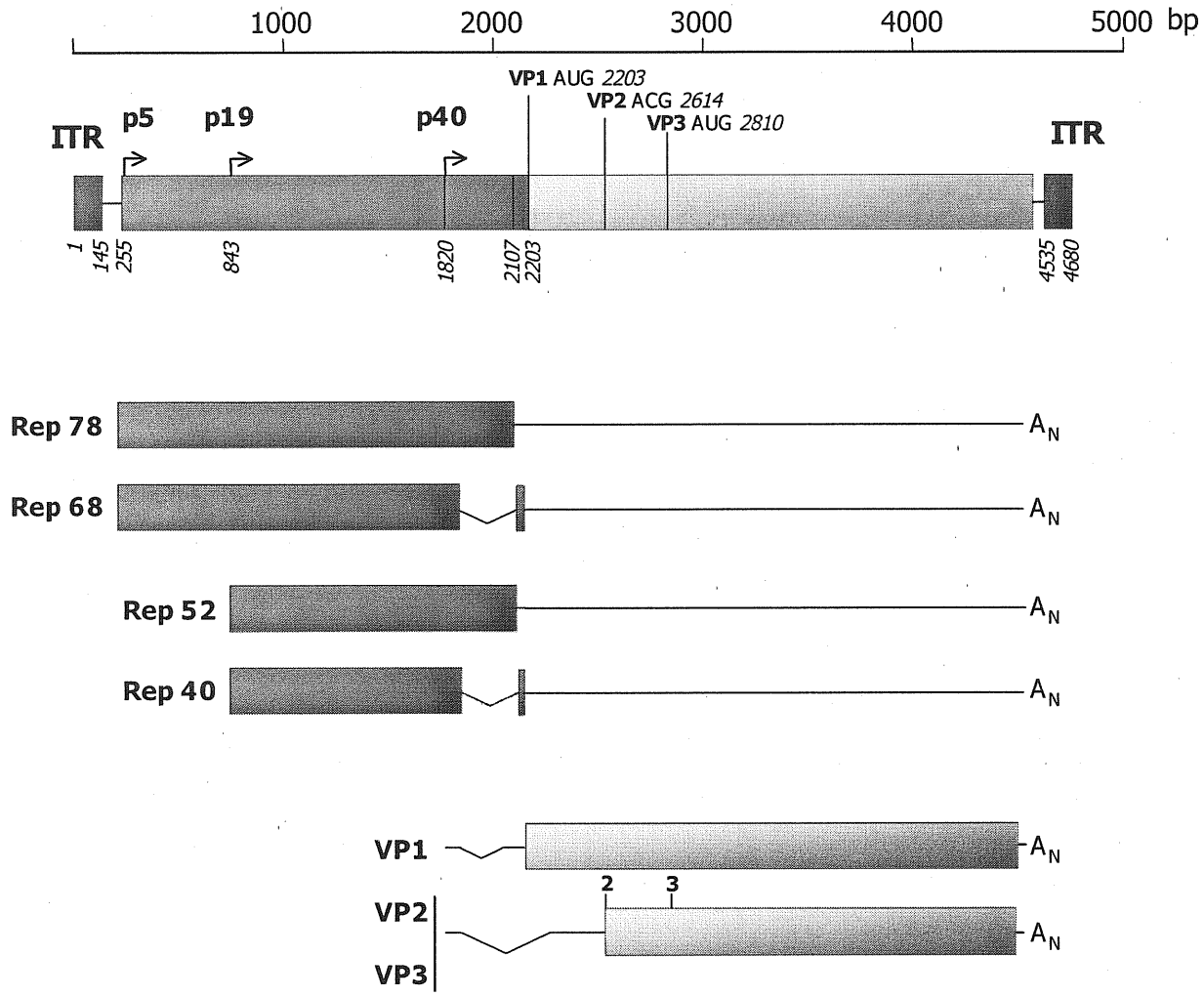


Fig 1.1) The AAV-2 genome and its transcripts. The main genetic elements of the AAV-2 genome, together with the transcripts produced from promoters p5, p19 and p40 are indicated. The positions of the inverted terminal repeats (ITR), of the promoters and of the start sites of Cap proteins are also represented.

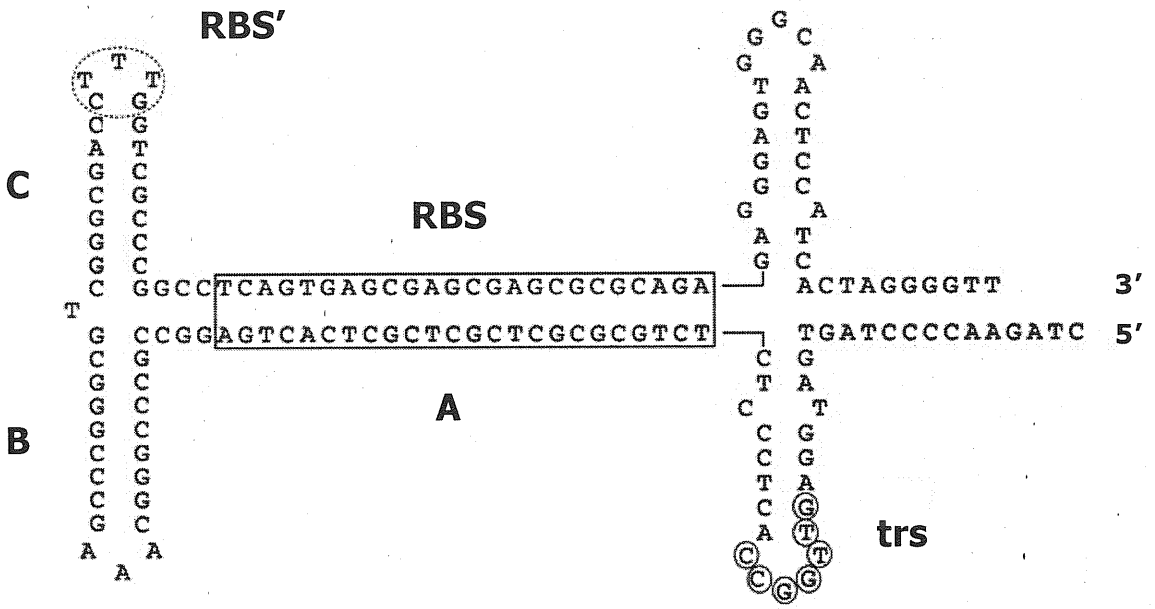


Fig 1.2) The AAV-2 ITR. The main features of the ITR are depicted: the T hairpin is formed by base-pairing maximization of the A,B and C internal palindromic sequences. Rep specifically binds the RBS sequence, which is boxed, and the RBS' sequence on the bulge of one of the two stems. Upon binding, Rep can also specifically cut DNA at the trs by extruding it in a hairpinned conformation, as shown. Adapted from (Brister and Muzyczka, 2000).

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 nick 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 mRNA (Green and Roeder, 1980; Laughlin et al., 1979) (See Fig 1.1). 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 alternative 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).

1.3 AAV-2 Rep proteins and their biochemical activities.

Rep78, Rep68, Rep 52 and Rep40 were named after their apparent molecular weight on SDS-PAGE gel. Various biochemical activities have been ascribed to the Rep proteins and now a great deal of information has been gathered on their function (Fig 1.3).

Rep68 and Rep78, which are 536 and 621 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 o Rep: Arg 107 and Arg 138 that make contacts with DNA bases whereas Lys 136 forms salt bridge with the phosphate backbone of DNA (Hickman et al., 2004). The RBS is the minimal requirement for Rep binding to the ITR but in the presence of the hairpin, that contains RBS', the efficiency of nicking increases up to 100 folds, (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, Tyr 156 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 (His 90 and His92), Glu83 and Asp412 (Hickman et al., 2002) (Urabe et al., 1999) (Yoon-Robarts and Linden, 2003) (Gavin et al., 1999). More recently, structural studies on the crystallized first 197 aa of AAV-5 Rep (alone or in the presence of RBS and RBS' oligos) have given 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

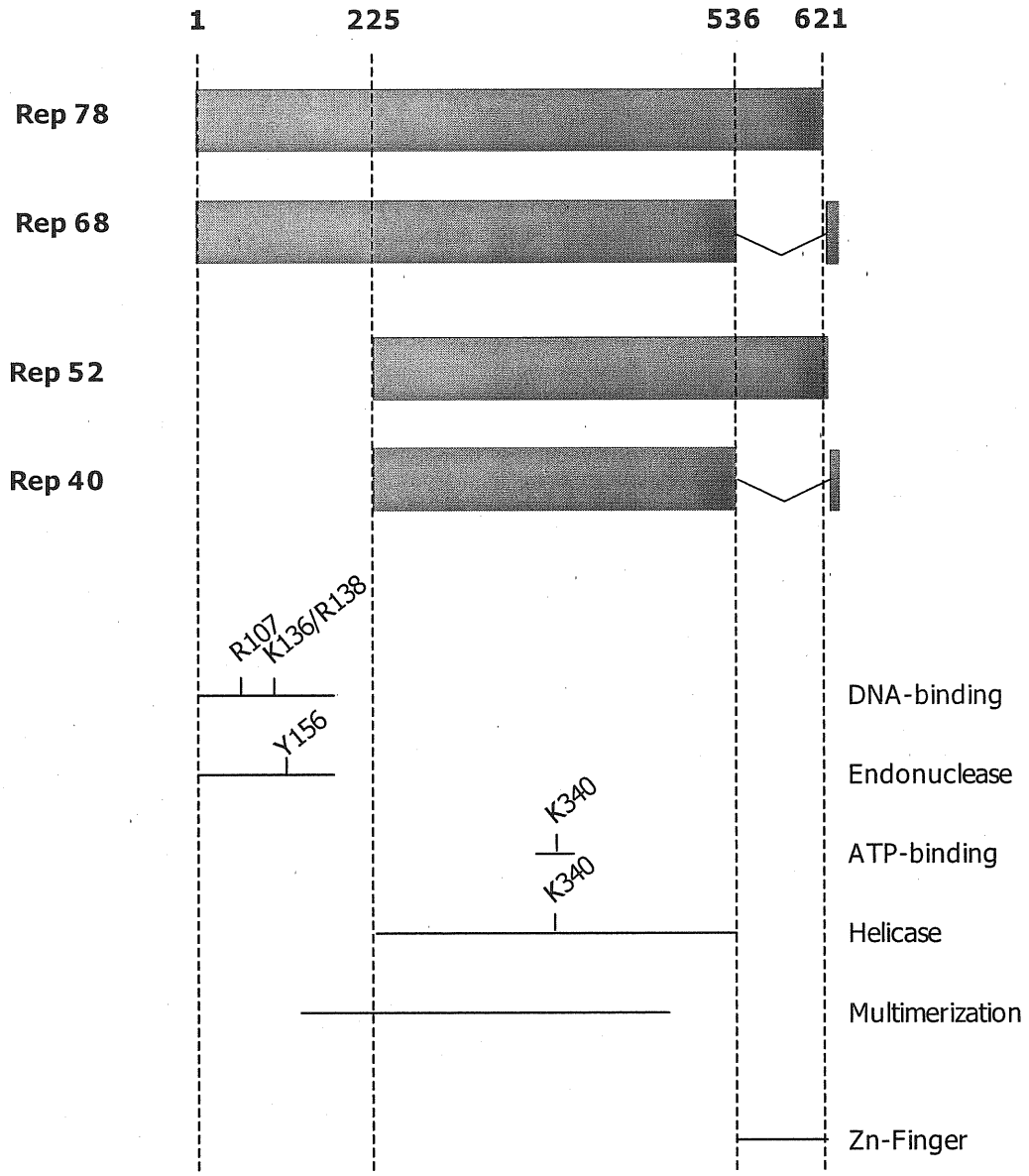


Fig. 1.3) Functional domains of AAV-2 Rep. Schematic representation of functional domains AAV-2 Rep proteins. The position of specific residues, necessary for the various biochemical activities mentioned on the right, is indicated.

also confirmed what had been observed in the past: Rep proteins bind RBS DNA as an 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 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). Resolution of the 3D structure of crystallized AAV-5 Rep40 by X-ray diffraction revealed that this AAV-5 protein constitutes the prototype of a new SF3 subgroup of the AAA^+ ATPase family (ATPases associated with diverse cellular activities extended family) (James et al., 2003).

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 G_2/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 PRKA and the inhibition of CREB-dependent transcription (Di Pasquale and Stacey, 1998).

1.4 AAV-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), Ad 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 evidences point out a role for HSV-1 ICP0 in 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.

1.5 AAV-2 replication.

AAV replicates through a strand discplacement 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 (Fig 1.4). This operation is carried on by Rep78 or Rep68 that bind the ITR and cut the terminal resolution site (trs) at position 124 only on the original strand, thus generating a free 3' OH on the same strand and transferring the "old" hairpin on 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 that 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 requires. Furthermore, a cell

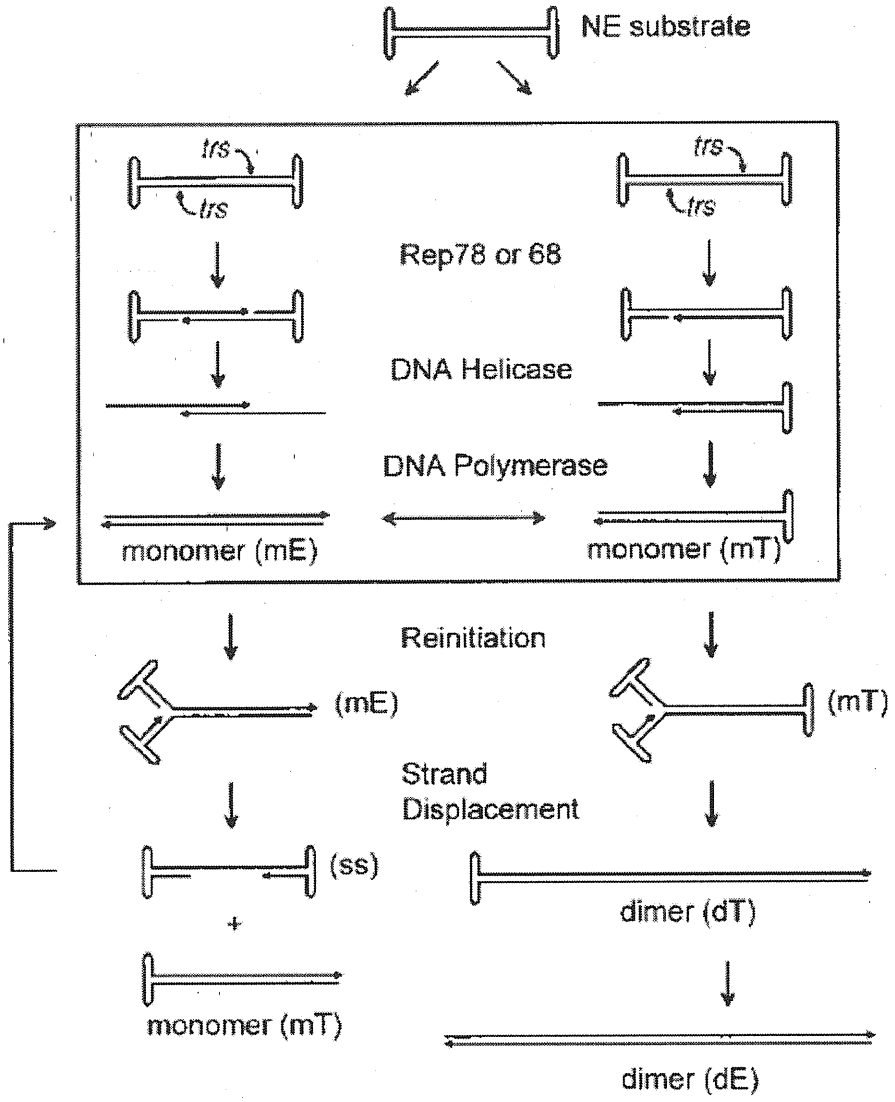


Fig 1.4) Diagram of AAV replication. The current model established for AAV replication is showed. NE: No end substrate; trs: terminal resolution site; mE:monomer extended form; mT: monomer turnaround; ss: single strand; dE: dimer extended; dT: dimer turnaround. Adapted from (Ni et al., 1998).

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 immunodepeletion 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 purifed HSV-1 proteins (UL5, UL8, UL29, UL30, UL42, and UL52) (Ward et al., 2001).

1.6 AAV-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). 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 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., 1996). 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 $2X10^5$ RBE are present throughout the genome (Young et al., 2000). Given that Rep binds all this 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).

1.7 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 Ad E1A gene is required for relieving Rep inhibition of the p5 and p19 promoters (Chang et al., 1989). Ad VA1 RNA, instead, is probably involved in facilitating the initiation of protein translation. An Adenovirus virus 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, Ad 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). Furthermore, E4ORF6 increases 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).

1.8 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 to influence the state of the host cell and to inhibit the replication of helper viruses.
First, Rep proteins have a biostatic effect 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 oncogens 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 citostatic activity can be correlated to the ability of inducing either a S-phase (Rep78) or a G_2/M (Rep68) cell cycle arrest by increasing the levels of phosphoryated 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, 1994b), c-H-ras (Batchu et al., 1994), HIV-1 LTR (Horer et al., 1995) and this work, 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). Further insights into this topic will be presented in this thesis.

Chapter 2: Materials and Methods.

2.1 Cell culture.

Human HEK 293, HeLa and U2OS (Human osteosarcoma cell line) cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) with Glutamax (Life Tecnologies, Inc.) supplemented with 10% fetal bovine serum (Life Tecnologies, Inc.) and gentamicin (100 μg/ml) at 37 °C in a humidified 93% air, 7% CO_2 incubator.

Transfections were performed by standard calcium phosphate co-precipitation method. Briefly, 24 hours before transfection 293 cells were plated at about 50% confluency. HBS 2X (50 mM Hepes pH 7.0, 280 mM NaCl, 10 mM KCl, Na_2HPO_4 1.5 mM, dextrose 12 mM,) was added drop wise to the DNA-$CaCl_2$ mix in a 15 ml conical tube with continuous bubbling. The total amount of DNA for each reaction was normalized adding empty vector. The mix was added directly onto cells. After 16 hrs cells were washed once in PBS and fresh medium was added.

2.2 Plasmids.

pcDNA3Rep68, pTKM32, pCMV16E2, pCDNA3DP1, pCDNA3E2F1, pGEX-Rep68N, pGEX-Rep40, pGEX-Tat86, pLTR-CAT, pcDNA3-Tat86, pcDNA3p300, pGL3TATAE2F6XLuc, pGEX-E2F1 and pLTR-Luc have been previously described (Cara et al., 1996; Marcello et al., 2000; Marzio et al., 1998; Marzio et al., 2000). pCMVβp300 and pHisRep68 were obtained by D.M. Livingstone (Dana Farber Cancer Institute, Boston, USA) and M. Linden (Mt. Sinai School of Medicine, New York, USA), respectively. HA-AMF1 and pGEX-AMF-1 were cloned by PCR using pcDNA3-AMF-1 (a kind gift of E. Androphy, Tuft University School of Medicine) as a template. pNLS-GFP was provided by M. Myers (Cold Spring Harbor Laboratories, New York, USA). Mammalian and bacterial expression vectors for the production of AMF-1 were generated by PCR amplifying AMF-1 ORF and was inserted as a BamHI-EcoRI fragment into pCMV-HA and pGEX4-T1(Promega), respectively. AMF-

1-GFP was obtained by PCR amplifying AMF-1 as a EcoRI-BamHI fragment that was then inserted in pQBI25/fN1 (Qiagen). HA-AMF-1 Δ11/75 lacks a.a. from 11 to 75, corresponding to the coiled coil region. This mutant was obtained by cutting pcDNA3AMF-1 with HindIII and re-ligating the digested plasmid. pFlag-HDAC3 was obtained by K. Kuhne, ICGEB, Trieste, Italy.

Flag-Rep68 and Flag-Rep40 were constructed as follows: the open reading frames for Rep68 and Rep40 were PCR amplified using FlagRep68fw and FlagRep40fw as forward primers (see table 2.1). Since the Rep68 and Rep40 share their C-terminal region, FlagRep68rw was used as reverse primer in both cases. The PCR products were cut with ClaI/KpnI and cloned into pFlag-CMV2 (Sigma, St. Louis, USA) cut ClaI/KpnI. The resulting construct has a Flag epitope cloned in frame at the 5' end of Rep ORF. Plasmid pHisRep68, a derivative of pET-16b (Novagen, Milwaukee, USA) used for the expression of N-terminally His-tagged Rep68, was obtained from M. Linden (Mt. Sinai School of Medicine, New York, USA) described (Marcello et al., 2000).

Specific primers (See table 2.1) were designed for amplification of the coding sequences of ANP32A and ANP32B from cDNA (A kind gift of Michael P. Myers, Cold Spring Harbor Laboratories, New York, USA). PCR products were cut XbaI/KpnI and cloned into pGCN (Provided by M. Myers, Cold Spring Harbor Laboratory, New York, USA) cut XbaI/KpnI to obtain pHAANP32A and pHAANP32B. These plasmids contain a N-terminal HA-tagged version of the ANP32A or of the ANP32B gene under the control of a CMV promoter.

Plasmids pcDNA3ANP32A and pcDNA3ANP32B, used for in vitro transcription-translation, were obtained by excising the ORFs from the HA vectors mentioned above as an XbaI/KpnI fragment and inserting them into pcDNA3 (Invitrogen, Carlsbad, California,USA) cut with the same couple of restriction enzymes.

The Swap ANP32 mutants were obtained as follows. We amplified the gene region coding for the N-terminus (a.a. 1-160) of ANP32A (primers: HAANP32Afw/HAANP321), the N-terminus (a.a. 1-160) of ANP32B (primers: HAANP32Bfw/HAANP323), the C-terminus (a.a. 160-251) of ANP32A (primers: HAANP322/HAANP32Arw) and the C-terminus (a.a. 160-251) of ANP32B (primers: HAANP324/HAANP32Brw). The primers were designed in order to allow the

Primer Name	Primer sequence (5'-3')
FlagRep68fw	CCATCGATATTGCCGGGGTTTTACGAGAT
FlagRep40fw	CCATCGATATTGGAGCTGGTCGGGTGGCT
FlagRep68rw	GGGGTACCTCAGAGAGAGTGTCCTCGAG
HAANP32Afw	GGCGGCTCTAGAATGGAGATGGGCAGACGGATTC
HAANP32Arw	GGCGGCGGTACCTTAGTCATCATCTTCTCCCTC
HAANP32Bfw	GGCGGCTCTAGAATGGACATGAAGAGGAGGATCC
HAANP32Brw	GGCGGCGGTACCTTAATCATCTTCTCCTTCATC
HAANP321	CATCCACCTCAGCATCCGAGTCAGGGGCCTCC
HAANP322	CTCAGATGCCGAGGGCTACGTGGAGGGCCTGG
HAANP323	CGTAGCCCTCGGCATCTGAGTCAGGTGCTTCC
HAANP324	CTCGGATGCTGAGGTGGATGGTGTGGATGAAG
HANP32DeltaCrw	GGCGGTACCTTAATCTGAGTCAGGTGCTTCC
pSuperANP32Afw	GATCCCCAGAAGAGCTTGGTGAAGAATTCAAGAGATTCTTCACCAAGCT CTTCTTTTTTGGAAA
pSuperANP32Arw	AGCTTTTCCAAAAAAGAAGAGCTTGGTGAAGAATCTCTTGAATTCTTCAC CAAGCTCTTCTGGG
pSuperANP32Bfw	GATCCCCAGAGGAGTTTGATGAAGAATTCAAGAGATTCTTCATCAAACTC CTCTTTTTTGGAAA
pSuperANP32Brw	AGCTTTTCCAAAAAAGAGGAGTTTGATGAAGAATCTCTTGAATTCTTCAT CAAACTCCTCTGGG
CMV-1	ACGGTAAACTGCCCACTTGG
CMV-2	CTTGGAAACCCCGTGAGTC

Tab 2.1) DNA oligos used in this study.

annealing of the ends of fragments amplified from ANP32A and ANP32B. These PCR fragments were then denatured, annealed and subjected to a single round of extension. Then they were used as a template for the second round of PCR using the external primers. These PCR products were then cut XbaI/KpnI and ligated into pGCN and pcDNA3 cut with the same couple of restriction enzymes.

Deletion mutants of the N-terminus of ANP32B were obtained by PCR using HAANP32Brw as the reverse primer and HANP32deltaN1fw, HANP32deltaN2fw and HANP32deltaN3fw as forward primer, respectively. These PCR products were then cut XbaI/KpnI and ligated into pGCN and pcDNA3 cut with the same restriction enzymes.

pDG helper plasmid for AAV2 vector production has been previously described (Grimm et al., 1998). pTR-UF5 (A kind gift of J. Samulski, Univ. of North Carolina at Chapel Hill, USA) contains the expression cassettes for the GFP reporter gene and for the neomicine resistance flanked by two AAV2 ITR.

2.3 Antibodies.

α-Rep polyclonal rabbit antiserum was a kind gift of J. Kleinschmidt (German Cancer Research Centre, Heidelberg, Germany). Mouse monoclonal α-Flag M2 antibody, mouse monoclonal anti α-tubulin, and Flag M2 agarose conjugated beads were purchased from Sigma (St. Louis, USA). Rat monoclonal α-HA high affinity (3F10) antibody was purchased from Roche Diagnostics. Mouse monoclonal α-KU70 (N3H10), mouse monoclonal α-KU80 (111) and mouse monoclonal α-DNA-PK_{cs} were purchased from NeoMarkers (Fremont, USA). The α-HDAC3 and α-GFP antibodies were purchased by St. Cruz Biotechnology (St. Cruz, California, USA).

2.4 Short interfering RNA.

To select the target sequences in the ANP32A and ANP32B genes for RNA interference we followed the criteria published by Reynolds and colleagues (Reynolds et al., 2004) and the Whitehead Institute (Cambridge, Massachusetts, USA) siRNA selection program was used (available at this website: http://jura.wi.mit.edu/siRNAext/) (Yuan et al., 2004). The algorithm used for screening was NARNAN6TN2HN5WN2, where N is any nucleotide, R is a purine, H is any nucleotide but G, and W is A or T. Furthermore, candidate sequences were filtered for a 30-52% GC content, the absence of strong secondary structures. To avoid off-target silencing a Blast search at NCBI was performed to exclude candidate target sequences that had less than two nucleotides mismatches with other human genes (i.e. nor ANP32A or ANP32B). Such analysis yielded these sequences: 5'- GAAGAAGAGCTTGGTGAAGAAGA-3' corresponding to nt 673-685 of the ANP32 mRNA (NM_006305), and 5'-GAAGAGGAGTTTGATGAAGAAGA-3' corresponding to nt 559-671 of the ANP32B mRNA (NM_006401). Two complementary DNA oligos (See table 2.1) were synthesized for each pSuper plasmid vector. They were denatured, annealed, phosphorylated and gel purified before being ligated into pSuper (Brummelkamp et al., 2002) cut BglI/HindIII. Single colonies were picked and DNA sequenced to confirm the presence of the desired correct sequence.

2.5 Flag-Rep68 protein complexes purification.

36 hrs after transfection 20 large culture dishes of HEK 293 cells (about $6x10^8$ cells) were washed once in PBS and lysed on ice in 1ml/dish of Lysis Buffer (20 mM Hepes pH 7.9, 150 mM NaCl, 0.5% NP40, 1 mM DTT, Protease inhibitors cocktail). The cell lysate was sonicated once for 15" at 30% output and then centrifuged 15' at 14000rpm at 4°C. An aliquot of the cleared lysate was kept as input, while the rest was incubated with 100 μl of packed and pre-equlibrated Flag M2 agarose beads. The lysate was then left on a rotating weel for 4 hrs at 4°C.

Beads were then rinsed twice with 40 ml of Lysis Buffer and then washed in the same buffer 3 times, 10' each. Immunocomplexes were eluted by adding 500 μg/ml of Flag peptide (Sigma, St. Louis, USA) in Lysis Buffer and incubated twice for 30' at 4 °C. The eluate was concentrated by standard TCA (Trichloroacetic acid) precipitation and resuspended in 30 μl SDS-PAGE Protein Loading buffer 1X.
The proteins were then run on a 10% SDS-PAGE gel and then stained with Zinc Stain following the protocol indicated by the producer (Biorad, Hercules, California, USA).

2.6 ESI-MS/MS sample preparation.

Bands were excised with a sharp blade and minced into small pieces (about 1 mm) with the help of a long gel-loading tip. De-staining was achieved by a 5' incubation in 100 mM EDTA pH 8.0. Gel pieces were washed 5x30', o/n, and then three more times in 1 ml of 20 mM $(NH_4)HCO_3$ pH 8.0, 50% Methanol. After the washing step, gel pieces were added with 100 μl of acetonitrile 100% and dried out in a speedvac.
In gel trypsin digestion was carried out by letting the gel pieces soak for 15' at 37°C in 10 μl of trypsin (Promega, Wisconsin, USA) 60 μg/ml dissolved in 20 mM $(NH_4)HCO_3$ pH 8.0. The gel pieces were then covered with pre-warmed 20 mM $(NH_4)HCO_3$ pH 8.0 and then incubated o/n at 37°C. The day after the supernatant was removed and put in a clean tube. Residual peptides were extracted from the gel by adding 5% HCOOH and sonicating for 20'.
Peptides present in the pooled supernatants were purified with a C18 Zip-Tip (Millipore), eluted in 10 μl of 95% acetonitrile and dried in a speedvac. The pellet was resuspended in 10 μl of BufferA.

2.7 Co-immunoprecipitations and MNase treatment.

36 hrs after transfection HEK 293 cells were washed once in PBS and lysed on ice in 1 ml/dish of Lysis Buffer (20 mM Hepes pH 7.9, 150 mM NaCl, 0.5% NP40, 1

mM DTT, Protease inhibitors cocktail). The cell lysate was sonicated once for 15" at 30% output and then centrifuged 15' at 14000rpm at 4°C. An aliquot of the cleared lysate was kept as input, while the rest was incubated with 10 μl of packed and pre-equlibrated Flag M2 agarose beads. The lysate was then left on a rotating wheel for 4 hrs at 4 °C. Beads were then rinsed twice with 1ml of Lysis Buffer and then washed in the same buffer 3 times, 10' each. Micrococcal nuclease (Sigma, St. Louis, Missouri, USA) treatment was performed essentially as described in (Lai and Herr, 1992). Briefly, beads were equilibrated once in Micrococcal Nuclease Buffer (10 mM Tris-HCl pH 7.0, 50 mM NaCl, 4 mM $CaCl_2$,) and then the enzyme was added to the beads. Beads were incubated for 30' at 37 °C and then washed twice with Lysis Buffer. Proteins were eluted in Laemli buffer 1X, boiled 5' at 37 °C and loaded on a 12% SDS-PAGE gel.

All the immunoprecipitation experiments described from chapter 3.1 to chapter 3.5 were performed essentially in the same way, excepts that we used RIPA-PBS (1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS in 1X phosphate buffered saline plus protease inhibitors) for cell lysis and bead washes.

2.8 *In vitro* transcription-translation.

The Sp6 *In vitro* coupled transcription translation kit (Promega, Madison, Wisconsin, USA) was used to radiolabel proteins with ^{35}S-Methionine. We followed the protocol provided by the manufacturer.

2.9 Recombinant protein production of HisRep68.

A starter culture of *E.Coli* BL21 (DE3) (Stratagene) cells carrying the pHisRep68 plasmid were grown o/n at 37 °C in TB (Terrific Broth) + Ampicillin 100 μg/ml. The culture was diluted 1:100 in 500 ml of fresh TB + Ampicillin 100 μg/ml and let it grow to an $O.D._{600}$ of 0.5-0.8. At this point bacteria were induced with IPTG 1 mM for 4 hrs at 30 °C, collected by centrifuging and resuspended in 10 ml of Lysis Buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% Glycerol, Protease inhibitors

cocktail, 10 mM Imidazole). Cells were sonicated 3x20" and Triton X-100 was added to a final concentration of 1%. The bacterial lysate was then clarified by centrifugation for 30' at 15000 rpm. 500 μl of pre-equilibrated Ni-Nta affinity beads (Qiagen, Germany) were added to the lysate and the mixture was incubated for 1 hr at 4°C on a rotating wheel. Beads were washed with 150 volumes of Lysis Buffer and then kept at –80 °C until further use.

2.10 *In vitro* protein binding assays.

Recombinant HisRep68 bound on Ni-Nta beads (about 1μg of protein per reaction) or beads alone were thawed on ice and equilibrated twice in DNase/RNase buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM $CaCl_2$, 10% Glycerol). To remove any contaminating DNA or RNA molecules DNase (1 Unit) and RNase (5 units) were added and the samples were incubated for 1 hr at 25 °C. Beads were washed once in 1 M NaCl, washed twice in Binding Buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% NP40, 20 mM β-Mercaptoethanol, 10 mM Imidazole) and then blocked for 30' at 4 °C in 1 μg/ml of BSA (Bovine Serum Albumin) in Binding Buffer. Beads were washed three more times with binding buffer and the *in vitro* transcribed and translated ^{35}S radiolabelled protein (about 300 cpm per reaction) was added. The binding reaction was carried on at 4 °C for three hours and then the beads were washed 5 times with Binding Buffer. At the end they were resuspended in SDS-PAGE Protein Loading buffer 1X. Eluted proteins were run on a on a 12% SDS-PAGE gel, Coomassie stained, and the gel was dried. Counts per minute were measured with a Phosphoimager apparatus (Packard), The percentage of bound proteins was calculated comparing the cpm of each sample with the cpm of 20% of the original input. In GST pull-down assays the protocol was essentially the one outlined above, except for the binding buffer had this formulation: 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 1 mM DTT.

2.11 AAV Replication assay.

For the replication assay and low molecular weight DNA was isolated by a modified HIRT protocol (Hirt, 1967). Briefly, 36 hrs after transfection HEK 293 cells were collected in the medium, centrifuged, washed once in PBS and resuspended in 200 μl of PBS. Cell lysis was achieved by adding 200 μl of Hirt Solution (1.2% SDS, 20 mM EDTA) and incubating for 15' at room temperature. Genomic DNA was precipitated by adding 100 μl NaCl 5M. After an incubation of 1 hr on ice, samples were centrifuged three times at 4°C and the pellet was discarded. The supernatant was precipitated with 0.6 volumes of isopropanol and washed once with freezer cold 70% Ethanol.

Samples were subsequently resuspended in 200 μl of Proteinase K buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.2% SDS). Proteinase K was added to a final concentration of 0.2 mg/ml and the reaction was performed for 2 hrs at 56 °C. DNA was extracted twice in phenol: chloroform: isoamilic alcool 25: 24: 1, and once in chloroform. The aqueous phase was ethanol precipitated and the DNA was resuspended in 30μl of ddH_2O and incubated for 20' at 68 °C with 0.02 Units of RNase. The solution was then divided in two equal aliquots and one of them was digested o/n at 37°C with 1 Unit of DpnI to remove input plasmid DNA. The other aliquot was kept as a control. DNA electrophoresis on a 0.7% TAE gel was performed as follows. DNA loading buffer 6X (60 mM Tris-HCl, 0.1% blue bromophenol, 2.5% gycerol) was added and the samples were heated at 65 °C for 5', and then put on ice for other 5' before loading on the gel. The purpose of this step was to reduce the possible smearing in the gel of single stranded rAAV genomes produced by replication.

The gel was incubated 30' in denaturing solution (0.5 N NaOH, 1.5 M NaCl), then 30' in neutralizing solution (0.5 N Tris-HCl pH 7.0, 1.5 M NaCl) and finally equilibrated 30' in 20X SSC (3 M NaCl, 0.4 M Sodium Citrate pH 7.0). The gel was blotted o/n using a Turboblotter apparatus (Schleicher and Schuell, Dessel, Germany) on a NytranN nylon transfer membrane (Schleicher and Schuell, Dessel, Germany). The membrane was then washed 5' in 2X SSC and air dried before UV crosslinking at 254 nm.

As a probe for hybridization, a 1kb XhoI restriction fragment of pTR-UF5 (Corresponding to the GFP expression cassette) was radiolabeled with α^{32}P-dCTP using the Ready to Go kit (Amersham-Pharmacia, Uppsala, Sweden) following the manifacturer instructions.

The membrane was pre-hybridized for 1 hr in Church buffer at 65 °C (0.5 M Sodium Phosphate pH 7.2, 7% SDS, 1mM EDTA) then the probe was added. The hybridization reaction was carried on at 65 °C. The day after, four round of washing with 40 mM Sodium Phosphate pH 7.2, 1% SDS were performed: 2X 5' at 65 °C, 1X 15' at 55 °C, 1X 1 hr at 65 °C. Results were acquired with a Cyclone (Packard). A Phosphoimager (Packard) was used for quantitative measurements.

2.12 AAV vector production.

36 hrs after transfection about $3X10^7$ HEK 293 transfected with pDG and pTR-UF5 were scraped and collected in 3 ml of Lysis Buffer (50 mM Hepes pH 7.6, 150 mM NaCl) and subjected to three cycle of freeze thaw to release the virions. The lysate was centrifuged 15' at 3000 rpm to remove cell debris and the supernatant was transferred to a clean tube. The residual pellet was resuspended in 2 ml of Lysis Buffer, frozen and thawed twice more and centrifuged 15' at 3000 rpm. The cleared lysates from the two cycles were pooled and precipitated by adding 0.33 volumes of saturated $(NH_4)_2SO_4$. After a 10' incubation on ice samples were centrifuged 30' at 14000 rpm, then the pellet was discarded and the supernatant was further precipitated by adding 0.66 volumes of saturated $(NH_4)_2SO_4$ and by incubating 10' on ice. Finally, The centrifuged supernatant was dyalized o/n against 5 l of PBS at 4 °C.

To titrate encapsidated AAV genomes samples were digested first with 10 U of Dnase for 1hr at 37 °C, boiled 2' at 95 °C to inactivate the enzyme, and then incubated for 1 hr at 56 °C with 133 μg/ml of Proteinase K, which was further inactivated for 5' at 95 °C.

Dnase/ProteinaseK digested samples were quantified by competitive PCR using a couple of primers (CMV-1 and CMV-2, see table 2.1) that amplify a 243 bp

fragment of the CMV promoter present in pTR-UF5. A 223 bp competitor was used in the PCR reaction (Zentilin et al., 2001).

2.13 Transcriptional assays.

U2OS cells were transfected by the calcium phosphate method with the appropriate reporter plasmid in the absence or in the presence of the expression plasmids indicated in each figure. CAT assays were routinely performed with 1-5 µg of total protein extract as estimated by the Bio-Rad protein assay (Biorad, Hercules, CA, USA). Following extraction with ethyl acetate, samples were analyzed by thin layer chromatography and quantified with a Packard Instant Imager. Luciferase assays were performed with a Dual Luciferase Reporter assay kit (Promega, Madison, WI, USA) following the producer's instructions.

2.14 Nuclear extracts preparation.

Nuclear extracts form HeLa cells were prepared as described in (Dignam et al., 1983).

2.15 Sequence alignment.

The primary sequence of human ANP32A and ANP32B was aligned with the T-COFFEE server provided by CH.EMBNET.ORG (Poirot et al., 2003).

2.16 Indirect Immunofluorescence.

24 hrs after transfection HeLa cells grown on 4-chamber slide were washed 3X in PBS and then fixed 10' at RT in paraformaldehyde 3.7%, rinsed 1X in PBS, permeabilized 10' in Triton X-100 1% in PBS, and blocked 30' in PBS/ 5% Fetal

Calf Serum/ 0.1% Tween 20. Slides were incubated in the same buffer with the appropriate primary and secondary antibodies 1 hr at 37 °C in a humidified chamber. After several washes slides were mounted with VectaShield (Vector Laboratories Inc.) and observed at an inverted fluorescence microscope.

Chapter 3: Results.

3.1 Rep inhibits HIV-1 Tat and hE2F1 transcriptional activation.

Previous work conducted in our laboratory demonstrated that Human Papilloma Virus 16 (HPV16) E2 protein recruits the cellular co-activator/histone acetyltransferase p300 to activate transcription and viral replication (Marcello et al., 2000). We were also able to show that AAV Rep proteins disrupt this physical interaction, thus resulting in repression of HPV E2 mediated transcription and replication.

Following these observations, we wondered whether AAV Rep could interfere with the recruitment of p300 by other viral and cellular transcriptional factors. The disruption of physical protein-protein interactions involved in several pathways of transcriptional activation would explain the wide repressive effects of Rep on a large number of promoters. Therefore, to test this hypothesis, we assessed the effects of Rep on the activity of two promoters regulated by p300/CBP, namely HIV-1 Tat (Benkirane et al., 1998; Hottiger and Nabel, 1998; Marzio et al., 1998) (Fig. 3.1a) and human E2F1 (Marzio et al., 2000).

Co-transfection of an HIV-1 Tat86 expressing plasmid with p300 resulted in a 3-fold enhancement of the expression of the reporter CAT gene driven by the viral LTR over Tat86 alone (Figure 3.1a, lane two and three). In this system, Rep68 completely inhibited p300/CBP-mediated transactivation of the LTR promoter to background levels (Figure 3.1a, lane four).

To verify whether Rep had a similar effect on E2F1 transcriptional activation, we expressed Rep together with E2F1 and pGL3TATA, a reporter plasmid that contains a luciferase reporter gene under the control of a minimal promoter downstream of six E2F1 DNA binding sites (Fig. 3.1b). As expected, this construct was activated by the concomitant expression of E2F1 and DP1, a cellular protein essential for E2F1 activation (Fig. 3.1b, lane two). As in the case of HIV-1 Tat, p300 was capable of enhancing the activity of E2F1 twice in this experimental

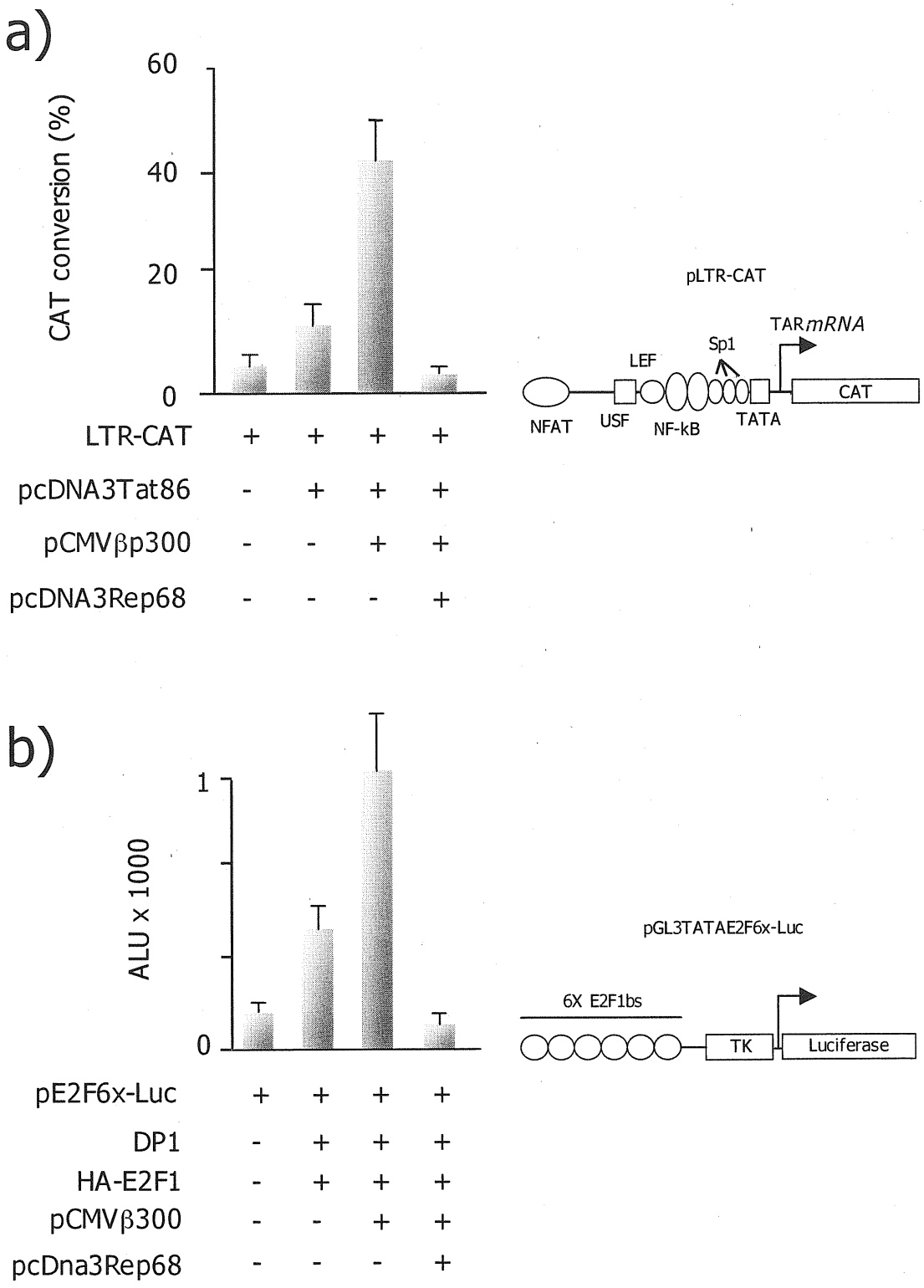


Fig. 3.1) Rep68 expression inhibits HIV-1 Tat and E2F1 activity. a) U2OS cells were transfected with the indicated combinations of plasmids. 24 hrs later CAT activity was measured. The organization of pLTR-CAT is depicted on the right. b) Same as a), but a synthetic E2F1 responsive promoter was used (pGL3TATAE2F6X-Luc, depicted on the right) and luciferase activity was measured.

conditions (Fig. 3.1b, lane three). Rep expression completely counteracted this activating effect of p300 (Fig. 3.1b, lane four). Collectively, the data presented in Figure 3.1 demonstrate that Rep has the property of inhibiting the activity of different cellular and viral transcription factors that share the p300 activation pathway. This effect is limited to this class of activators, because, in our experimental conditions, Rep68 did not alter the transcription levels of a control CMV-driven reporter (Marcello et al., 2000), thus demonstrating that Rep does not have an aspecific down-modulation effect on transcription.

3.2 Rep disrupts the physical interaction between transcriptional activators and p300.

In the case of HPV16 E2, we could demonstrate that Rep acted on this transcription factor by disrupting its physical binding with p300 (Marcello et al., 2000). Thus, we hypothesized that Rep might have an effect on protein-protein interactions occurring between E2F1 and p300, as well as between Tat and p300. To tackle this problem, *in vitro* transcribed and translated p300 was incubated with recombinant GST-Tat86 (Fig. 3.2a) or with GST-E2F1 (Fig. 3.2b). As previously reported, these two transcriptional activators were capable of retaining p300 on agarose beads (Marzio et al., 1998; Marzio et al., 2000). The physical interactions between both factors and the co-activator p300 were disrupted by adding increasing amounts of recombinant HisRep68 (Fig. 3.2a and Fig. 3.2b, lanes three, four and five). Thus, the data shown in Figure 3.2 provide a molecular explanation for the functional activity of Rep on the transcriptional activation of E2F1 and HIV Tat previously decribed.

From the data shown so far we can conclude that either Rep targets each transcription factor independently, or that Rep acts on a common cellular co-factor. However, the first hypothesis appears rather unlikely, given the large differences in primary sequence and origin of the three transcription factors (cellular E2F-1, HPV16 E2 and HIV-1 Tat).

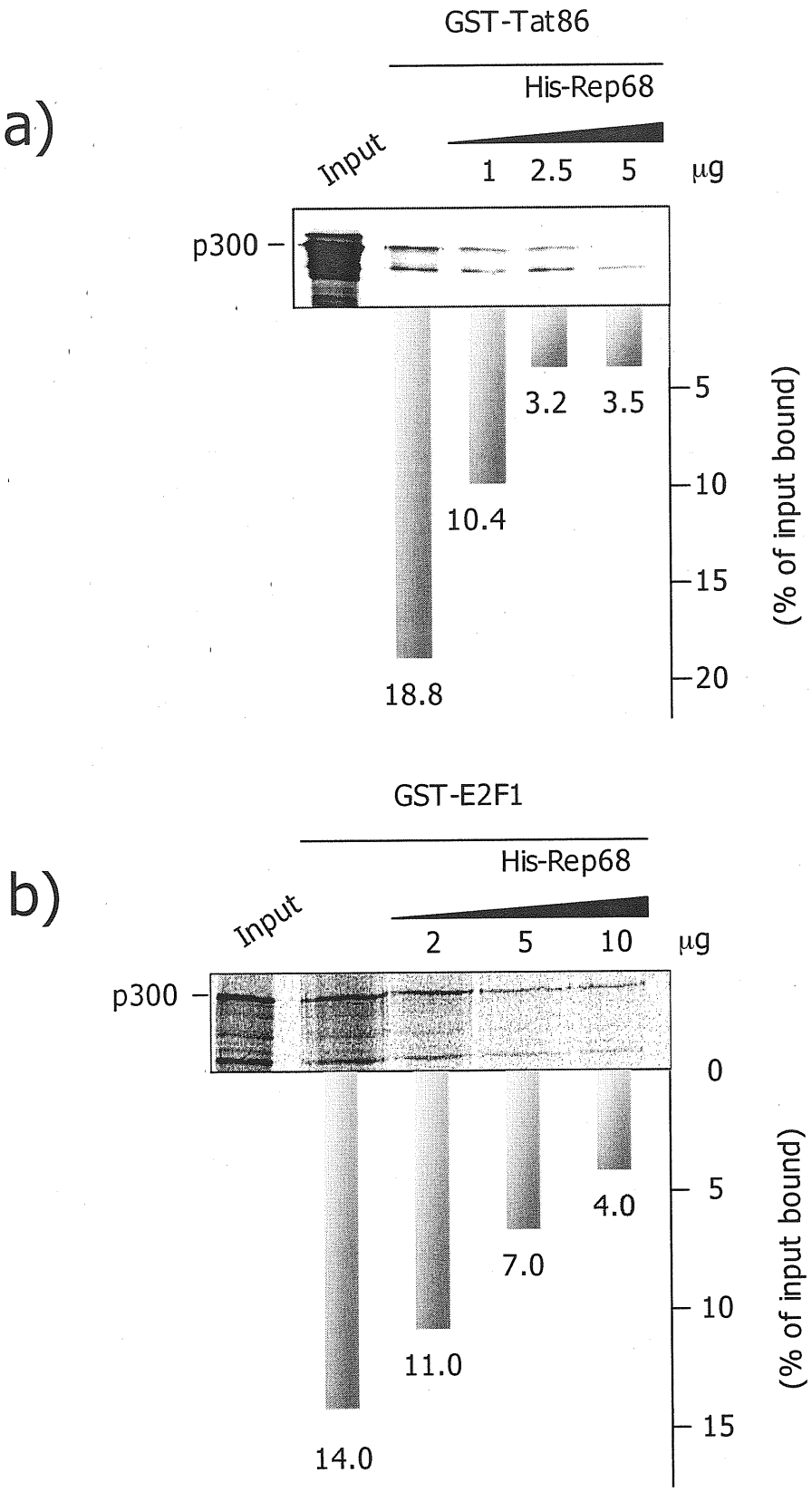


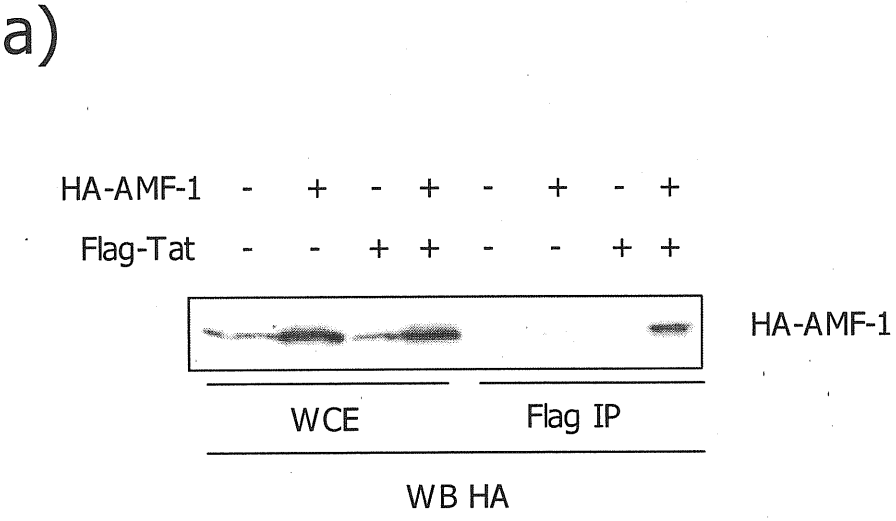
Fig. 3.2) Rep68 disrupts the binding between p300 and transcriptional activators. a) GST-Tat of HIV-1 bound on glutathione-agarose beads was incubated with *in vitro* transcribed and translated, ^{35}S methionine labeled, p300 in the presence of increasing amounts of recombinant HisRep68. The level of bound p300 (most upper band visible in the autoradiogram) was measured as the quantity of radioactivity that was retained on the GST-Tat beads and expressed as percentage of the input. b) Same as a) but GST-E2F1 was used instead.

3.3 AMF-1 is a protein partner for HIV-1 Tat and hE2F1.

While searching for a cellular protein common to all these transcription pathways, Androphy and collaborators identified AMF-1 (activation-domain modulatory factor 1), a cellular cofactor for p300 that is recruited by bovine papillomavirus (BPV) E2 to activate transcription (Breiding et al., 1997; Peng et al., 2000). This protein, also known as Gps2 (for G protein pathway suppressor 2) appears to be a general transcription factor targeted by several cellular proteins such as p53, HTLV-1 Tax and HPV E6 (Degenhardt and Silverstein, 2001; Jin et al., 1997; Peng et al., 2001). Intriguingly, all the factors mentioned above are also partners of p300. Thus we speculated that, being associated with many p300-binding proteins, AMF-1 might work as a general cofactor in this transcriptional activation pathway. To confirm this hypothesis we first had to demonstrate that AMF-1 was also a protein partner for the p300-dependent transcription factors analyzed in this study.

After Flag immunoprecipitations in HEK 293 cells, we could observe that HA-AMF-1 interacts with Flag-Tat when co-transfected (Fig. 3.3a, lane four), but not when one of them is absent (Fig. 3.3a, lane two and three). We could also observe the binding between HA-AMF-1 and GFP-E2F1 *in vivo* by co-immunoprecipitation (Fig. 3.3b, lane four).

To corroborate these results and to determine the AMF-1 domain involved in the interactions, we conducted *in vitro* binding experiments, in which *in vitro* transcribed and translated, ^{35}S labeled, AMF-1 was incubated with different GST fusion recombinant proteins produced in *E.coli*. We observed that GST-Tat is capable of binding both full-length AMF-1 (Fig. 3.4a, third lane) and the deletion mutant AMFΔ11/75 (Fig. 3.4a, lane six), which lacks residues 11-75, a region predicted to form coiled-coils involved in protein-protein interactions (Chun et al., 2000). Thus, from these experimental evidences we inferred that the coiled-coil domain of AMF-1 was not involved in the interactions between GST-Tat86 and AMF-1. The same conclusions could be also drawn for the interaction between GST-E2F1 and AMF-1 (Fig. 3.4b, lanes three and six).



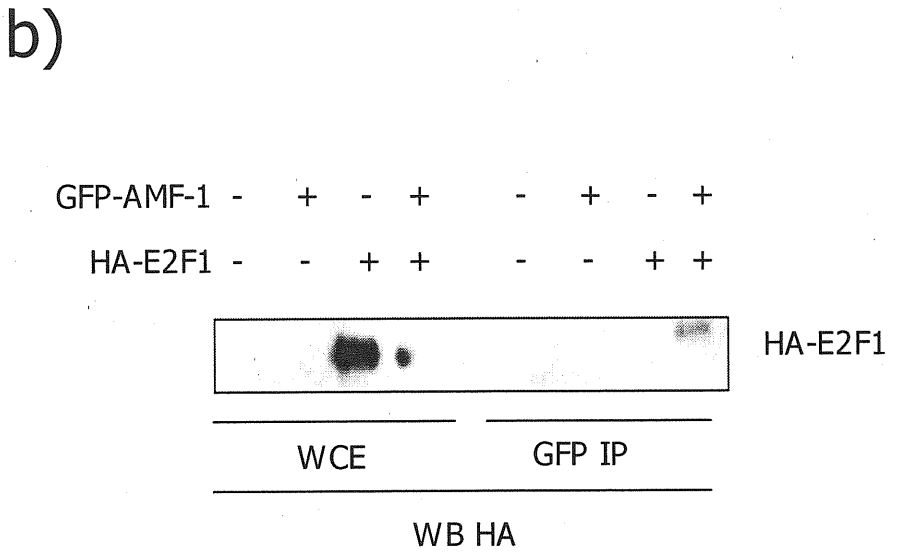


Fig. 3.3) AMF-1 binds HIV-1 Tat and hE2F1 *in vivo*. a) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with the indicated antibodies. Immunoprecipitates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure. b) Same as a). WCE: whole cell extract.

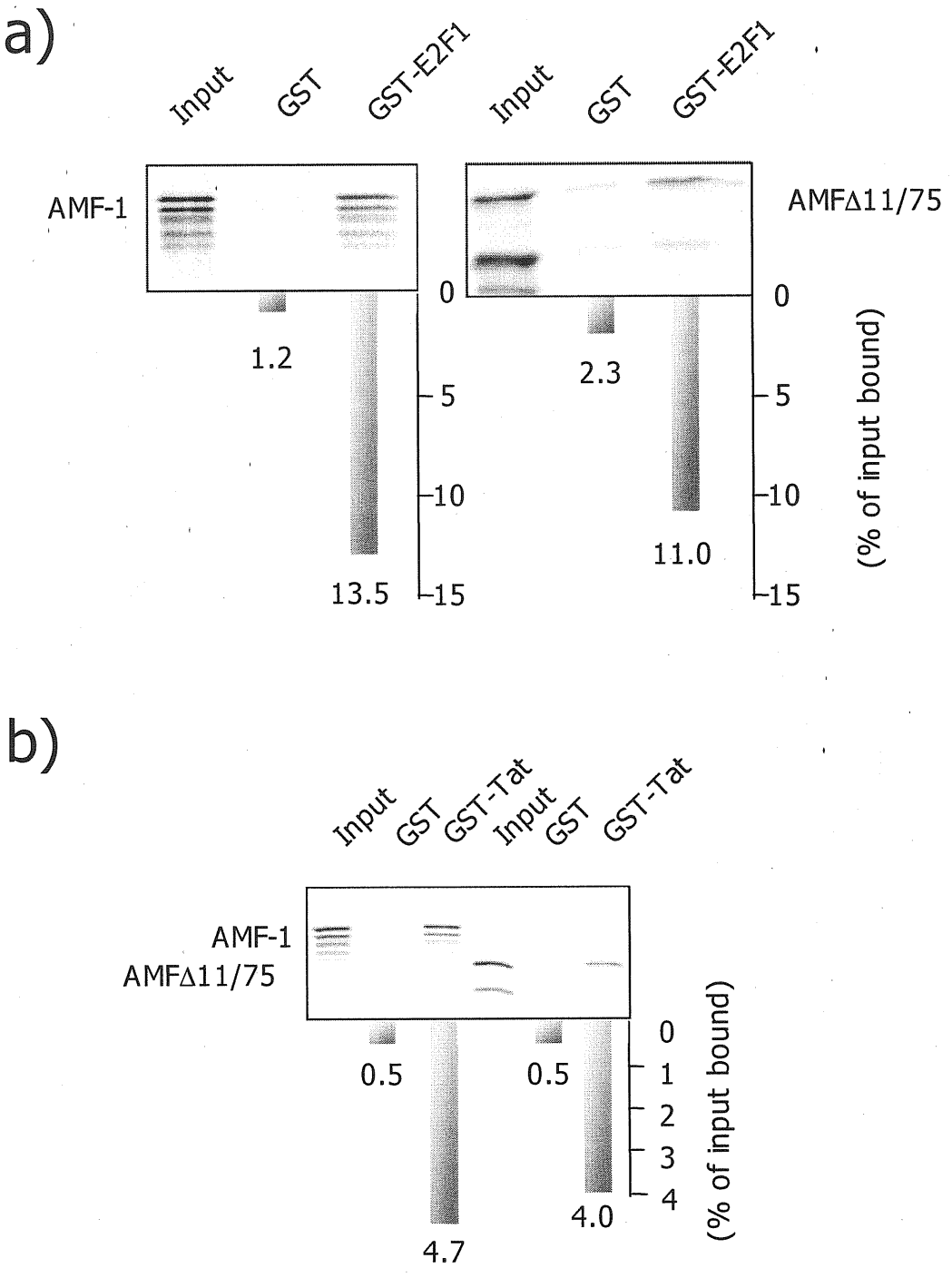


Fig. 3.4) AMF-1 binds HIV-1 Tat and E2F1 *in vitro*. a) GST-E2F1 bound on glutathione-agarose beads was incubated with either *in vitro* transcribed and translated ^{35}S methionine labeled AMF-1 or a mutant that lacks the coiled coil region (AMFΔ11/75). The level of bound AMF-1 or AMFΔ11/75 was measured as the amount of radioactivity that was retained on the GST-E2F1 beads and expressed as percentage of the input. b) Same as a) but GST-Tat was used.

3.4 Role of AMF-1 in HIV-1 Tat and E2F1 transcription.

What is the role of AMF-1 on transcriptional activity of E2F1 and Tat? The physical interaction between AMF-1 and the two transcriptional activators (Fig. 3.3 and 3.4) suggested that AMF-1 might function as a transcriptional regulator of HIV-1 Tat and hE2F1. Transcriptional assays conducted in the presence of transfected HA-AMF-1 suggested that this was indeed the case. In fact, the transcriptional activity of Tat on the LTR promoter was enhanced 3.5 times by the concomitant cotransfection of increasing quantities of AMF-1. HA-AMF-1 had a similar effect on the E2F1 synthetic responsive promoter depicted in Figure 3.1. We could observe a ~2 fold increase in transcription levels compared to E2F1 alone when HA-AMF-1 was added to the transfection mix (Fig 3.5b). We would like to underline that these observations are well in agreement with previous works that demonstrated a positive effect of AMF-1 on BPV E2 and p53 activity (Peng et al., 2000; Peng et al., 2001).

These results, in addition to experimental evidences presented by Peng and colleagues (Peng et al., 2000) point out a role for AMF-1 as a general transcriptional cofactor positioned between p300 and interacting transcriptional activators. As a consequence we speculated that Rep might repress transcription of several promoters by directly targeting AMF-1, thus inducing to the disruption of the complicated network of interactions that normally leads to transcriptional activation.

3.5 Rep binds AMF-1 and disrupts its binding with p300.

To confirm the above mentioned hypothesys we first demonstrated that Rep binds AMF-1 *in vivo* and *in vitro*. The results of these experiments are shown in Figure 3.6a. HA-tagged AMF-1 did indeed co-immunoprecipitate with Rep68 in HEK 293 cells (Fig. 3.6a, lane two). Furthermore, in vitro binding assays with recombinant GST-Rep proteins and in vitro translated AMF-1 demonstrated that the C-terminal part of Rep (corresponding to Rep40, aa 225-536 of Rep68) was the region

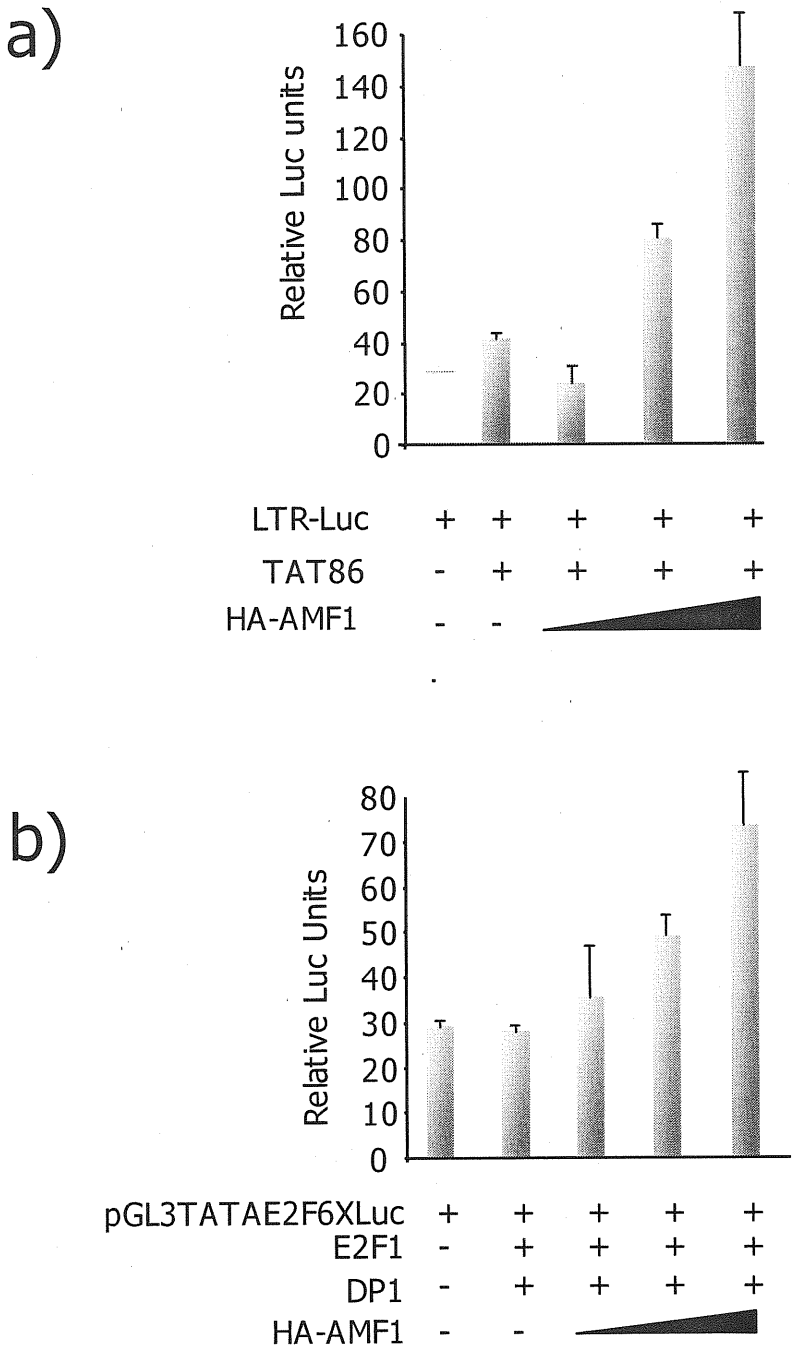


Fig. 3.5) AMF-1 co-activates HIV-1 Tat and E2F1 activity. a) U2OS cells were transfected with a plasmid containing the Luciferase reporter gene under the control of the HIV-1 LTR, plus a combination of the indicated plasmids. 24 hrs later Luciferase activity was measured. b) Same as a), but the pGL3TATAE2F6XLuc promoter was used.

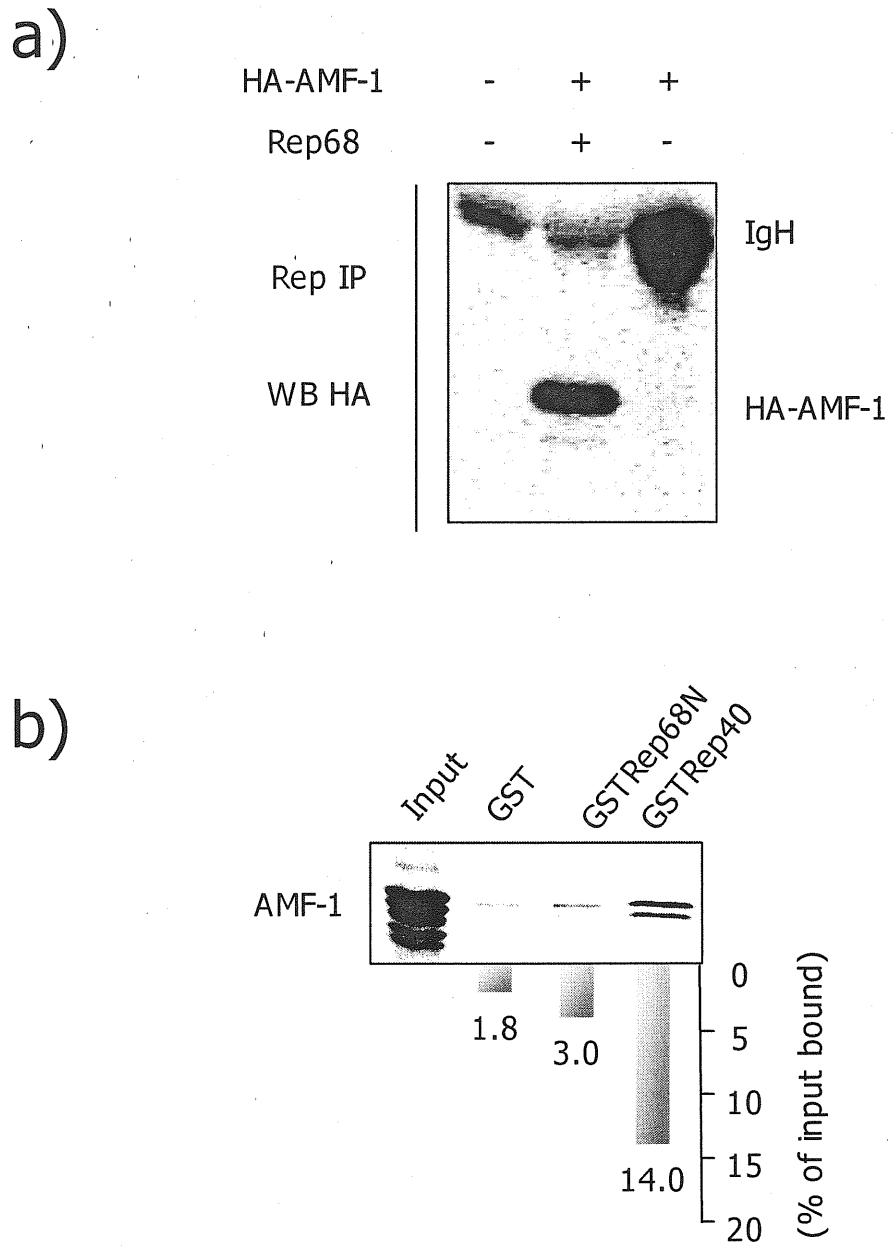


Fig. 3.6) Rep40 binds AMF-1 *in vivo* and *in vitro*. a) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with an anti-Rep antibody. Immunoprecipitates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with 3F10 anti-HA antibody. b) Either GST-Rep68N or GST-Rep40 bound on glutathione-agarose beads were incubated with *in vitro* transcribed and translated ^{35}S methionine labeled AMF-1. The level of bound AMF-1 was measured as the quantity of radioactivity that was retained on the GST-Rep beads and expressed as percentage of the input.

involved in this interaction (Fig. 3.6b, lane four), while the N-terminus (aa 1-224, Fig. 3.6b lane three) was not responsible for the binding. This last piece of data is well in agreement with our previous results regarding transcriptional inhibition mediated by Rep. In fact, this activity was ascribed to Rep40 alone (Marcello et al., 2000).

At this point, we reasoned that, being able to bind to AMF-1, Rep should have also been able to disrupt the interaction of AMF-1 with p300. As a confirmation of this hypothesis we observed that GST-AMF-1 does indeed bind to *in vitro* transcribed and translated p300 (Fig. 3.7a lane two), but this interaction was almost completely abolished by adding increasing amounts of HisRep68 in the reaction (Fig 3.7a). By performing a similar experiment, but probing the AMF-1/HPV16 E2 interaction instead (Fig 3.7b), we could demonstrate that HisRep68 did not have any significant effect on the interactions of these two proteins. This last finding demonstrates two important features of the protein-protein disruption mediated by Rep: a) it is a very specific mechanism limited to the binding of AMF-1 to p300 and b) it preserves the AMF-1/activator interaction intact.

3.6 Rep binds human HDAC3.

We have demonstrated that Rep binds AMF-1 (Fig. 3.6) and disrupts it binding with p300 (Fig 3.7). Roeder and collaborators recently reported the presence of AMF-1 in the N-CoR (Nuclear Corepressor) complex that include, beside AMF-1, N-CoR, TBL1, TBL1R and the histone deactelyase HDAC3 (Zhang et al., 2002). This complex is endowed with deacetylase activity and can inhibit transcription of of several cellular promoters (Perissi et al., 2004). We were then appealed by the hypothesis that might achieve repression of promoters by associating with HDAC3. Co-immunoprecipiation and in vitro binding experiments confirmed this hypothesys (Fig. 3.8). As it is possible to see from Fig. 3.8a lane three, a significant amount of endogenous HDAC3 from a HeLa nuclear extract was bound by HisRep68 immobilized on Ni-Nta beads. This result was confirmed *in vivo* by co-immunoprecipitating Rep68 in 293 cells together with FLAG-HDAC3 (Fig. 3.8b, lane 4).

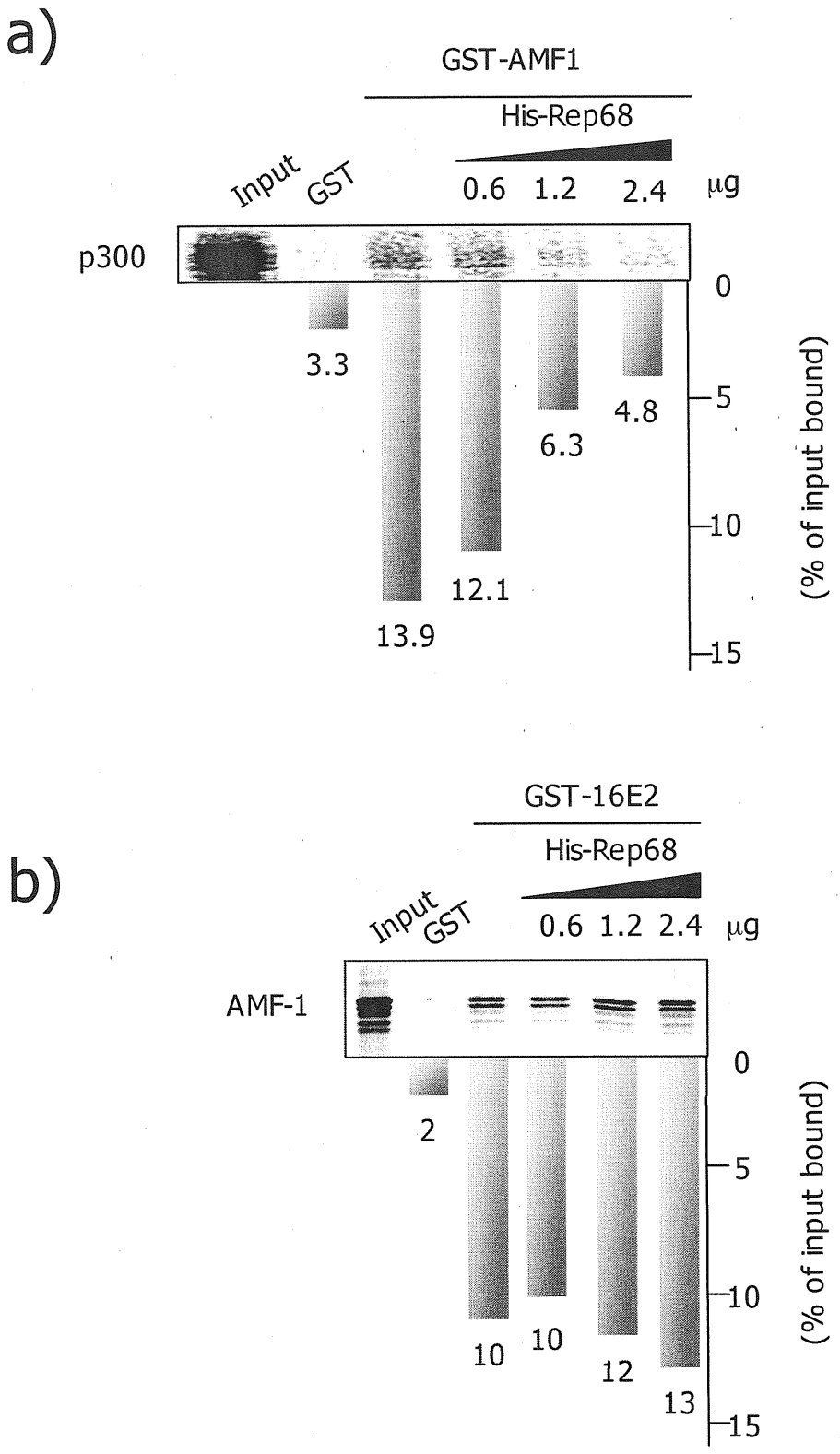


Fig. 3.7) Rep68 disrupts the binding between p300 and AMF-1 but not between AMF-1 and HPV16 E2. a) GST-AMF-1 bound on glutathione-agarose beads was incubated with *in vitro* transcribed and translated ^{35}S methionine labeled p300 in the presence of increasing quantities of recombinant HisRep68. The level of bound p300 was measured as the quantity of radioactivity that was retained on the GST-Tat beads and expressed as percentage of the input. b) Same as a) but GST-16E2 and *in vitro* transcribed and translated ^{35}S methionine labeled AMF-1 were used.

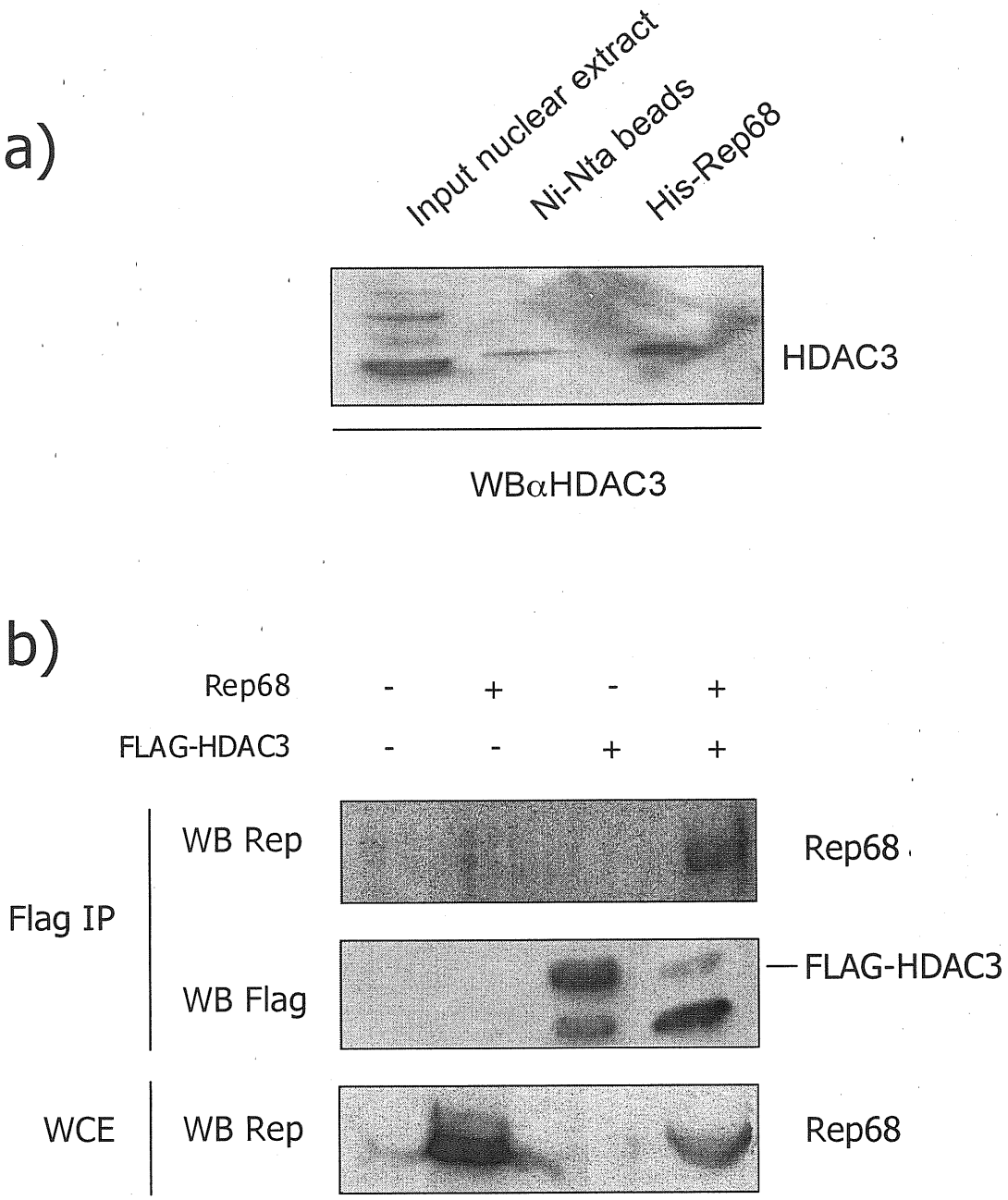


Fig. 3.8) Rep68 binds HDAC3 *in vitro* and *in vivo*. a) HisRep68 immobilized on Ni-Nta agarose beads was incubated with HeLa nuclear extracts. Bound proteins were run on a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Bound HDAC3 was visualized with specific antibodies. b) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with M2 anti-Flag agarose beads. Immunoprecipitates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure. WCE: whole cell extract.

In summary, from this set of data we can envisage a working model in which p300 acts as a platform capable of bridging components of the basal transcription machinery to chromatin remodeling complexes by the formation of a complex network of protein-protein interactions. Cellular transcription factors such as E2F-1 and viral proteins such as HIV Tat and HPV16 E2 can recruit p300 through the cellular cofactor AMF-1. AAV Rep destabilizes the interaction between AMF-1 and p300, thus inhibiting transcriptional activation. Furthermore, the association of Rep in human cells with HDAC3, probably through direct binding to AMF-1, is indicative of the involvement of the N-CoR complex in Rep mediated transcriptional repression. The implications of this working hypothesis will be discussed in Chapter 4. This model might help explaining the observed inhibition of HIV ad HPV replication by Rep (Antoni et al., 1991; Hermonat, 1994a). Furthermore, at the cellular level, the down-modulation of E2F-1 activity by Rep correlates with the AAV-mediated inhibition of cell proliferation.

3.7 Tagged proteomics of Flag-Rep68.

The Rep proteins play fundamental roles at every step of the AAV replication cycle and, moreover, they impinge on many cellular pathways, such as cell cycle regulation, apoptosis, DNA damage repair and transcription (See the Introduction chapter). While it is conceivable that several of these functions directly depend on the biochemical properties of Rep itself, it is likely that the outcome of AAV viral infection is also regulated by functional interactions between Rep and factors of the host cell. In fact, previous evidences have shown that Rep interacts with cellular proteins such as the transcriptional co-activator PC4 (Weger et al., 1999), the cell cycle regulator Rb (Batchu et al., 2002), the non histone chromosomal protein HMGB1 (Costello et al., 1997), p53 (Batchu et al., 1999) and more recently with the ssDNA binding protein RPA (Stracker et al., 2004). Each of these interactions have been shown to regulate various aspects of the AAV life cycle. Nevertheless, a comprehensive identification of Rep-containing protein complexes *in vivo* (i.e. in mammalian cell culture) has not been tackled so far. For this reason

tagged proteomics seemed to be the most appropriate and innovative approach to discover new Rep-interacting factors *in vivo*.

3.7.1 Flag-Rep68 localizes in the cell nucleus and is functionally active.

With this aim in mind, we chose to construct an N-terminal Flag epitope tagged version of Rep68 (The Rep isoform that we used for previous studies, (Marcello et al., 2000)) under the control of the CMV promoter.
Upon transfection in HeLa cells and staining with the M2 monoclonal α-Flag antibody, Flag-Rep68 localized in the cell nucleus (Fig. 3.9a), as already reported by several other laboratories (Stracker et al., 2004). Moreover, Flag-Rep68 was functional since it could promote the replication of a rAAV vector in vivo (Fig 3.9b, lane three). In fact, upon co-infection with Adenovirus-5 and co-transfection with the rAAV vector pTR-UF5, Flag-Rep68 was capable of generating DpnI resistant bands corresponding to the AAV replication intermediates RF1 and RF2 (monomer and dimer, respectively).

3.7.2 Purification and 1D SDS PAGE analysis of FlagRep68-associated complexes in 293 cells.

Cleared lysates from FlagRep68 HEK 293 transfected cells, as well as mock cells transfected with empty vector, were immunoprecipitated with agarose beads conjugated to the M2 Flag antibody, washed several times and subsequently eluted with Flag peptide (See Material and Methods chapter). Eluted protein complexes were concentrated by TCA precipitation and loaded on a 10% SDS-PAGE gel and stained with Zinc Stain (Fig. 3.10). From the gel picture it is possible to see that in the lane corresponding to immunocomplexes purificated from Flag-Rep68 transfected cells we could determine the presence of five major specific protein bands that were named p400, p90, p70, p68 and p60 according to their relative molecular mass on the gel. Further visual analysis of the gel (see enlarged

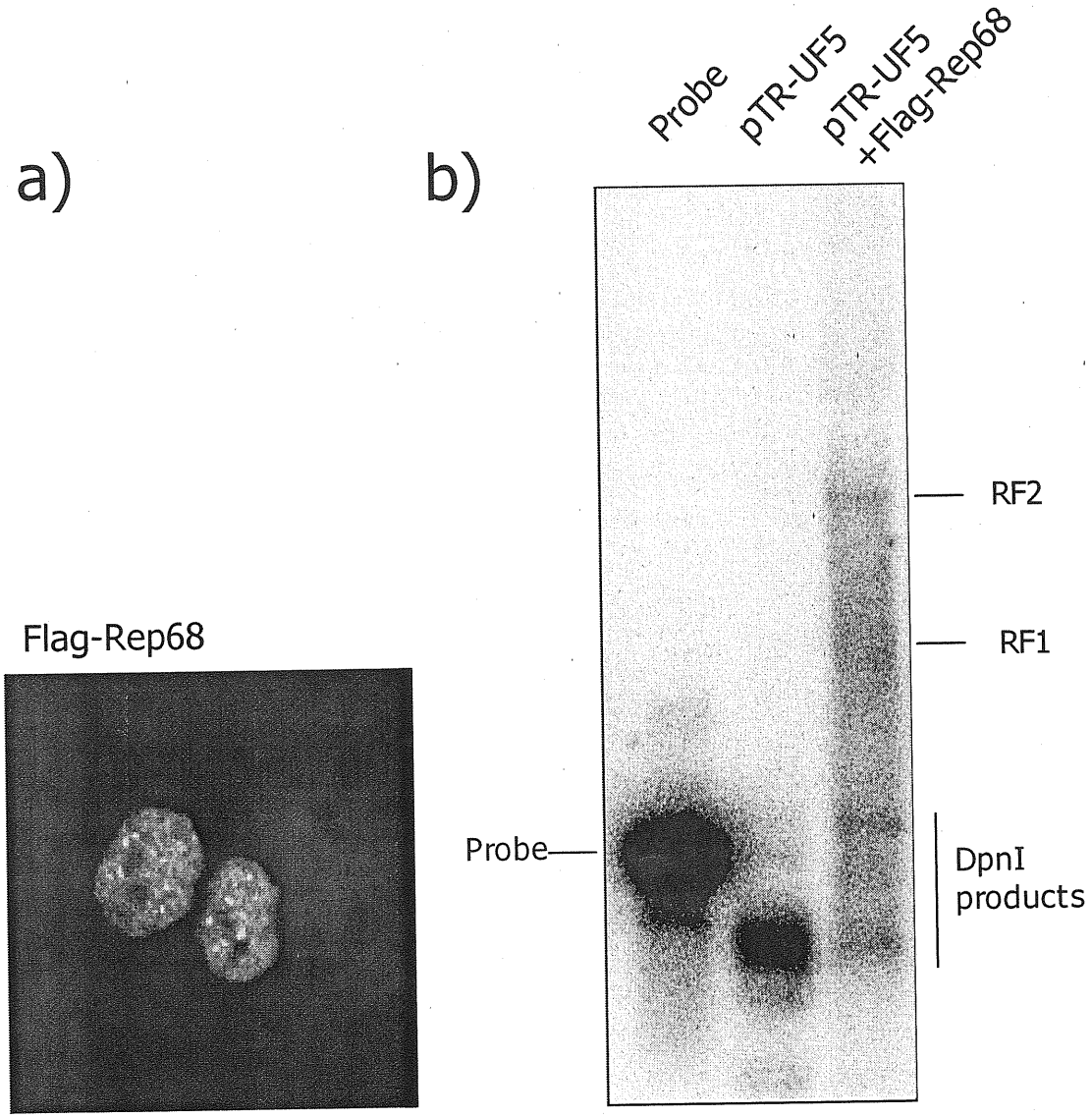


Fig. 3.9) Flag-Rep68 localizes to the nucleus and is functional in AAV replication. a) HeLa cells were transfected with Flag-Rep68. 36 hrs after transfection cells were fixed in paraformaldehyde and stained with M2 anti-Flag antibody. The secondary antibody used was 488-Alexa anti mouse (Molecular Probes). b) HEK 293 cells were transfected with the indicated combination of plasmids. After 16 hrs cells were infected with Ad-5 and collected after further 24 hrs. Hirt extracted DNA was digested with DpnI, run on a 0.7% TAE agarose gel, and transferred to a nylon membrane. Specific AAV replication bands were identified by using a 1kb fragment of XhoI-digested pTR-UF5 as a probe. Replication intermediates RF1 and RF2 are indicated, as well as the positive control for hybridization obtained by loading the probe itself on a separate lane.

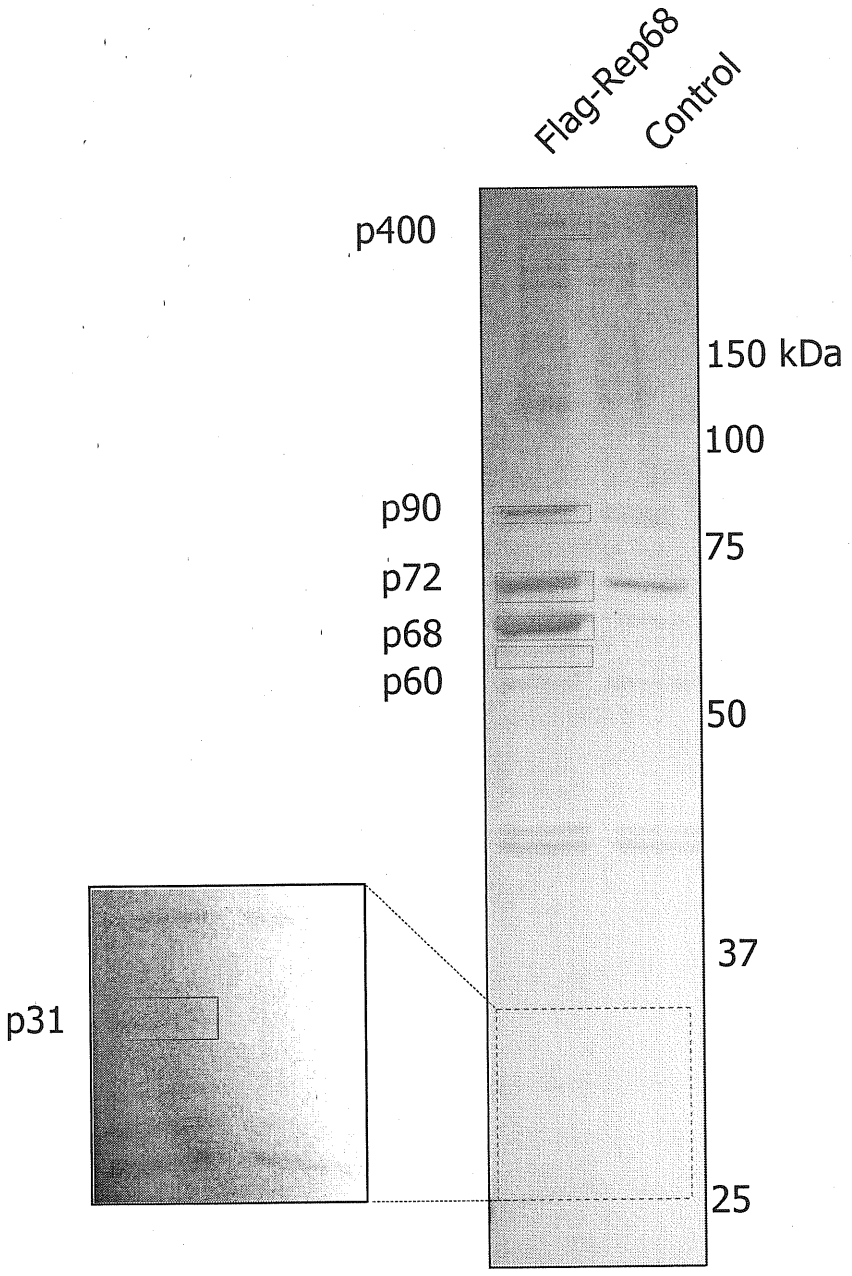


Fig. 3.10) Flag-Rep68 co-immunoprecipitates with several cellular proteins. HEK 293 cells were either transfected with an empty vector or with Flag-Rep68. After 36 hrs cell lysates were immunoprecipitated with M2 anti-Flag agarose beads. Specific Flag-Rep68 protein complexes were eluted with Flag peptide and run on a 10 % SDS-PAGE gel that was later stained with Zinc Stain. Specific protein bands boxed in red in the FlagRep68 lane were excised and their identity was determined by ESI-MS/MS (See Material and Methods chapter). The enlarged box represents the lower part of the gel. The two lower bands in the box that are not boxed in red were identified as ribosomal proteins and ignored for subsequent analysis (Data not shown). Marker molecular weights are indicated on the right.

box of Fig 3.10) revealed the presence of a less intense protein band, named p31, migrating at about 31 kDa, which was present in the Flag-Rep68 lane but not in the mock transfected control lane. Bands present also in the control lane were not taken in consideration, except for the unspecific band present in the control lane and co-migrating with p70 (Fig. 3.10).

3.7.3 Identification of Rep-interacting proteins by Mass Spectrometry.

To determine the identity of the Flag-Rep68 interacting proteins we excised protein bands from the gel and subjected them to sample preparation for Mass Spectrometry analysis (See Material and Methods chapter, and for a recent review on this subject see (Steen and Mann, 2004)). The peptides obtained by in-gel trypsin digestion were purified and analyzed by ESI-MS/MS.

Mass spectra were collected and interpreted by the software (Sonar MS/MS, Genomic Solutions), which then compared the peptide sequences obtained by fragmentation with a translated, non-redundant NCBI database of gene sequences. For each identification, the Expect value, a measure of statistical significance based on the length and the quality of the match, was calculated by the software. The Expect value is a parameter that describes the number of hits one can expect just by chance when searching a database of a particular size. This means that the lower the E-value, or the closer it is to the 0, the more significant the match is.

By analysis with ESI-MS/MS we could identify p400 as the catalytic subunit of the DNA-PK complex (DNA-Pkcs) (Accession#: NP_008835), p90 as Ku80 (Accession#: NP_066964), p72 as Ku70 (Accession#: NP_001460), p68 as AAV-2 Rep68 (Accession#: 040500), p60 as HSP60 (Accession#: NP_001469) and p31 as ANP32B (Accession#: NP_006392).

We were extremely confident in the quality of these identifications, because: a) the predicted masses for these proteins are well in accordance with their apparent molecular weights measured on the SDS-PAGE gel (Fig. 3.10); b) the Expect values for all the matches were far below $1X10^{-4}$ (Which can be considered as a

	Peptide sequence	Protein name (MW)	e-value
p400	3218-MEVQEQEEDISSLIR-3233	DNA-PKcs (468KDa)	$2.9X10^{-83}$
	2504-DVLIQGLIDENPGLQLIIR-2522		
	855-VVQMLGSLGGQINK-868		
	3303-TVSLLDENNVSSYLSK-3318		
	1988-YNFPVEVEVPMER-2000		
	3993-SDPGLLTNTMDVFVK-4007		
	839-NLSSNEAISLEEIR-852		
	2787-HSSNEAISLEEIR-2800		
	3051-LLLQGEADQSLLTFDIK-3067		
	3814-DLLLNTMSQEEK-3825		
	3325-DQNILLGTTYR-3335		
	914-VTELALTASDR-924		
	3475-YPEETLSLMKT-3485		
	747-AYVPALQMAFK-757		
	2777-HGDLPDIQIK-2786		
	2133-LGNPIVPLNIR-2143		
	4024-GGSWIQEINVAEK-4036		
	2075-LGLPGDEVDNK-2715		
	1618-LATTILQHWK-1627		
	1076-LGASLAFNNIYR-1087		
	2637-ATQQQHDFTLTQTADGR-2653		
	3890-MSTSPEAFLALR-3901		
	811-NNWEVSALSR-820		
p90	82-HLMLPDFDLLEDIESK-97	KU80 (83KDa)	$8.4X10^{-31}$
	131-HIEIFTDLSSR-141		
	569-TEQGGAHFSVSSLAEGSVTSVGSVNPAENFR-599		
	145-SQLDIIIHSLK-155		
	503-ELPPIQQHIWNMLNPPAEVTTL-525		
	355-FFMGNQVLK-363		
	546-DQVTAQEIFQDNHEDGPTAK-565		
	401-ANPQVGVAFPHIK-413		
	709-DKPSGDTAAVFEEGGDVDDLLDMI-733		
	535-TLFPLIEAK-543		
p72	101-NIYVLQELDNPGAK-114	KU70(70KDa)	$9.9X10^{-38}$
	219-DIISIAEDEDLR-230		
	266-DIVISVGIYNLVQK-279		
	81-DLLAVVFYGTEK-92		
	195-DTGIFLDLMHLK-206		
	405-NIPPYFVALVPQEEELDDQK-424		
	302-TFNTSTGGLLLPSDTK-317		
	596-KQELLEALTK-605		
	475-SDSFENPVLQQHFR-488		
p68	279-TAPDYLVGQQPVEDISSNR-297	Rep68 (60KDa)	$4.3X10^{-20}$
	73-APEALFFVQFEK-84		
	241-QWIQEDQASYSFNAASNSR-260		
	373-MVIWWEEGK-381		
	123-GIEPTLPNWFAVTK-136		

p60	292-APGFGDNR-299	HSP60 (61KDa)	
	51-TVIIEQSWGSPK-62		
	212-GYISPYFINTSK-223		
	196-TLNDELEIIEGMK-208		
	241-ISSIQSIVPALEIANAHR-258		
	361-IQEIIEQLDVTTSEYEK-377		
	484-IMQSSSEVGYDAMAGDFVNMVEK-506		

p31	138-LLPQLTYLDGYDR-150	ANP32B (29KDa)	$1.9X10^{-7}$
	76-IFGGLDMLAEK-86		
	87-LPNLTHLNLSGNK-99		
	102-DISTLEPLKK-111		
	102-DISTLEPLK-110		

Tab. 3.1) Sequences of the peptides identified by ESI-MS/MS. The position of the peptide relative to the original protein sequence is indicated. The predicted molecular mass of the hits and the E-value relative to the identification of the protein is showed.

threshold value); c) we were able to identify at least five tryptic peptides for every protein band that we cut from the gel (Table 3.1) and d) every protein was independently identified in at least two repetitions of the same experiment.
HSP60 (Heat Shock Protein 60) is a member of the Heat Shock Protein family and it has molecular chaperonine activity toward misfolded proteins. Heat shock proteins are very abundant in the cell and prone to unspecific protein binding. The band migrating at about 70 kDa in the control lane was identified as human PRMT5 (Protein arginine methyltransferase 5) (Accession#: 001460). Both HSP60 and PRMT5 were not considered for further analysis.

3.8 Rep68 interaction with the DNA-PK complex is dependent on DNA.

DNA-PKcs, Ku80 and Ku70 are the three components of the human DNA-PK complex. Current knowledge indicates that the DNA-PK complex plays a pivotal role in repairing DNA double strand breaks by homologous end joining (NHEJ). DNA-PK initially recognizes and binds to damaged DNA and then targets the other repair activities to the site of DNA damage. (For a recent review see (Bakkenist and Kastan, 2004)).
Previous evidences from different laboratories suggested a role for the DNA-PK complex in the processing of AAV genomes upon cell infection. Ku80 was detected bound on the genome of an AAV vector after infection, and $Ku80^{-/-}$ cells were found to be more permissive to AAV vector transduction (Zentilin et al., 2001). Beside, it was also shown that DNA-PK influences the fate of AAV infection by modulating AAV genomes circularization (Duan et al., 2003; Song et al., 2001) and integration (Song et al., 2004) *in vivo*. Thus we were intrigued by the possibility that the AAV Rep/DNA-PK interaction played a biological role in the context of AAV infection, and for this reason we performed standard co-immunoprecipitation experiments to confirm this physical interaction *in vivo*. The results of these experiments are shown in Figure 3.11.

As a confirmation of the results presented in Figure 3.10, Flag immunoprecipitation in the absence of Micrococcal Nuclease was able to pull-down endogenous DNA-PKcs, Ku80 and Ku70 from Flag-Rep68 transfected HEK 293 cells (Fig.3.11a, lane five), but not in mock transfected cells (Fig.3.11a, lane one). In contrast Flag-Rep40 (encompassing a.a. 225-526 of Rep68) did not co-immunoprecipitate any of the proteins mentioned above (Fig.3.11a, lane three). Since Ku is a very well known protein that binds DNA ends and structural DNA elements at high affinity, and Rep is a binding protein as well, we wanted to test whether this molecular interaction was mediated by the presence of DNA. For this reason the Flag immunoprecipitates were treated with micrococcal nuclease in co-immunoprecipitation experiments, following the protocol already established by Lai *et al* (Lai and Herr, 1992). By treating immunoprecipitates with Micrococcal Nuclease, we were not able to detect the presence of any of the members of the DNA-PK complex associated with Rep68 (Fig. 3.11a, lane six). Flag western blot on the immunoprecipitates confirmed that equal quantities of Flag-Rep68 and Flag-Rep were immunoprecipitated in both conditions, thus excluding the possibility of protease contaminations in the nuclease stock solution. Western blot against the Ku70 subunit of the complex on total cell lysates confirmed that the input amount of endogenous proteins used for immunoprecipitation was the same in every sample. In summary, the results of this experiments indicate that the physical interaction between Rep68 and the members of the DNA-PK complex is unlikely to take place *in vivo.* In this respect, however, it should be pointed out that this conclusion does not simplistically indicate that the detected interactions a mere result of DNA contamination of the samples used for co-immunoprecipitation. As we mentioned above, DNA damage and repair pathways (included NHEJ) are involved in AAV genome processing and integration. We cannot therefore exclude the possibility that DNA indeed triggers an interaction between Rep and DNA-PK. This experimental finding might have important biological implications for AAV replication cycle and further experiments will be needed to gain further insight into this possibility.

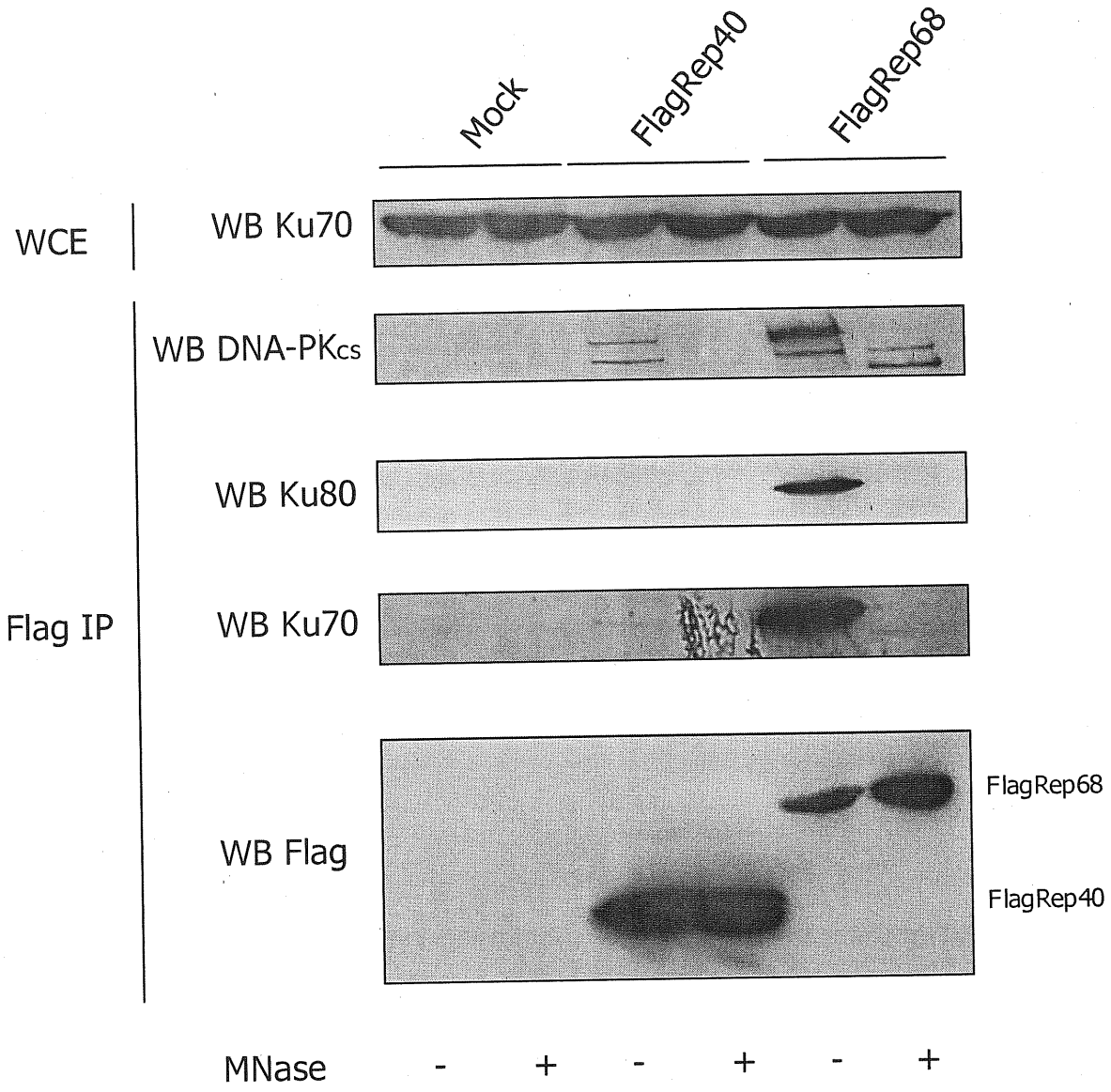


Fig. 3.11) Flag-Rep68 binds the DNA-PK complex in a DNA dependent manner. a) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with M2 anti-Flag agarose beads. Immunoprecipitates were treated with micrococcal nuclease, loaded on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure. WCE: whole cell extract. MNase: micrococcal nuclease.

3.9 Flag-Rep68 specifically interacts with HA-ANP32B *in vivo* and *in vitro.*

The p31 protein that we identified as ANP32B belongs to the Acidic Nuclear Protein 32 family and it has been described in the literature as a nuclear phosphoprotein involved in the regulation of several cellular pathways, many of which are also regulated by Rep (See the Introduction Chapter).

We were convinced of the specificity of these physical interactions for two reasons: first, it was identified twice in Flag-Rep68 immunocomplexes (Fig. 3.10, Tab. 3.1 and data not shown); and second, it was not present in the mock control lane, as assessed by Mass spectrometric analysis (not shown).

We also want to point out that we were not able to identify ANP32A, another member of the same protein family, in the Flag-Rep68 immunoprecipitates. In addition, three of the tryptic peptides that we identified for p31 (see Tab. 3.1) were specific for ANP32B, as boxed in red in the alignment between human ANP32A and ANP32B (Figure 3.12a). Consequently, Rep68 seemed to display a remarkable degree of specificity in the interaction with ANP32B, even though human ANP32A and ANP32B are 70% identical in terms of amino-acidic sequence and share several structural domains (Figure 3.12a).

To confirm our findings, and to study the properties of ANP32A and ANP32B, their open reading frames were PCR amplified with specific primers from a human cDNA library and cloned into a mammalian expression vector fused in frame to an influenza virus haemoagglutinin (HA) tag. The constructs were verified by DNA sequencing and shown to contain the expected sequences.

We next asked whether the Rep and ANP proteins are present in the same sub-cellular compartment. To test this hypothesis, pHA-ANP32A and pHA-ANP32B were transfected into human HeLa cells, which were consequently fixed in paraformaldehyde and stained with a monoclonal anti-HA antibody (Figure 3.12b). The over-expressed constructs showed a diffuse nuclear staining, with the exclusion of the nucleoli. On this side, Rep is a nuclear protein that carries on most of its functions in the nucleus ((Hunter and Samulski, 1992), (Stracker et al., 2004) and Fig. 3.9).

a)

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sp|P39687|A32A_HUMAN   MEMGRRIHLELRNRTPSDVKELVLDNSRSNEGKLEGLTDEFEELEFLSTINVGLTSIANL
sp|Q92688|A32B_HUMAN   MDMKRRIHLELRNRTPAAVRELVLDNCKSNDGKIEGLTAEFVNLEFLSLINVGLISVSNL
                       *:* ************: *:*******.:**:**:**** ** :***** ***** *::**

sp|P39687|A32A_HUMAN   PKLNKLKKLELSDNRVSGGLEVLAEKCPNLTHLNLSGNKIKDLSTIEPLKKLENLKSLDL
sp|Q92688|A32B_HUMAN   PKLPKLKKLELSENRIFGGLDMLAEKLPNLTHLNLSGNKLKDISTLEPLKKLECLKSLDL
                       *** ********:**: ***::**** ************:**:**:******* ******

sp|P39687|A32A_HUMAN   FNCEVTNLNDYRENVFKLLPQLTYLDGYDRDDKEAPDSDAEGYVEGLDDEEEDE------
sp|Q92688|A32B_HUMAN   FNCEVTNLNDYRESVFKLLPQLTYLDGYDREDQEAPDSDAE--VDGVDEEEEDEEGEDEE
                       *************.*****************:*:********  *:*:*:*:*****

sp|P39687|A32A_HUMAN   DEEEYDEDAQVVEDEEDEDEEEEGEEEDVSGEEEEDEEGYNDGEVDDEEDEEELGEEERG
sp|Q92688|A32B_HUMAN   DEDDEDGEEEEFDEEDDEDEDVEGDEDDDEVSEEEEEFGLDEEDEDEDEDEEE-EEGGKG
                       **:: * : : .::*:****: **:*:* . .***:* * :: : *::*****  *  :*

sp|P39687|A32A_HUMAN   QKRKREPEDEGEDDD
sp|Q92688|A32B_HUMAN   EKRKRETDDEGEDD-
                       :******.:******
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b)

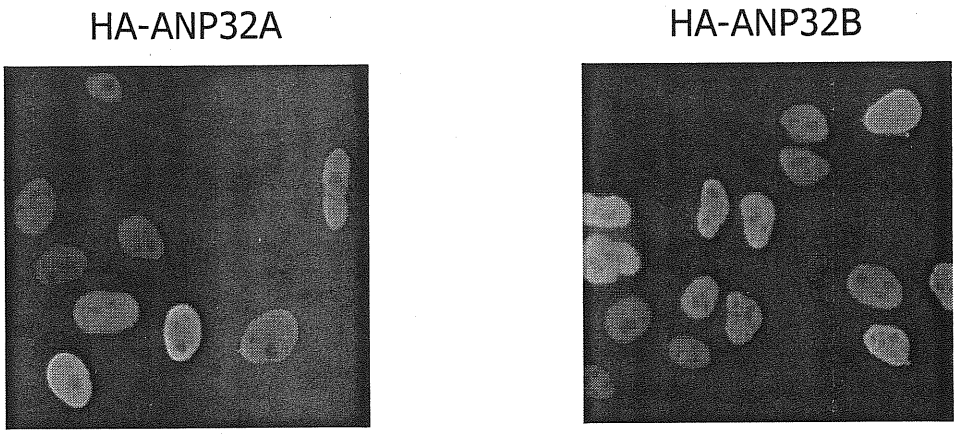


Fig. 3.12) Alignment of human ANP32A and ANP32B and their sub-cellular localization. a) The protein sequences of ANP32A and ANP32B were aligned using T-COFFEE (See Material and Methods chapter). Asterisks denote identity whereas dots denote homology between two residues in the same position. The peptides of ANP32B sequenced by ESI-MS/MS (See Table 3.1) are boxed in red. b) HeLa cells were trnsfected with either pHA-ANP32A or pHA-ANP32B. 36 hrs after transfection cells were fixed in paraformaldehyde and stained with 3F10 anti HA Antibody. The secondary antibody used was Alexa-555 anti rat (Molecular Probes).

As a confirmation of the results presented in Figure 3.10, co-immunoprecipitation experiments with HA-tagged ANP proteins and Flag-Rep68 were performed in the presence of micrococcal nuclease, in order to exclude any possible role of DNA in mediating the physical interaction between the proteins under exam. The anti-HA western blot on Flag immunoprecipitates showed that Flag-Rep68 was capable of pulling down HA-ANP32B (Fig. 3.13a, lane six) and, to a much lesser extent, HA-ANP32A (Fig. 3.13a, lane five). This might be due either to a lower affinity of Rep68 for ANP32A compared to ANP32B, or by the fact that binding of Rep68 to the former is not direct and is mediated by another factor. Control Western blots on the same immunoprecipitates and on the cell lysate show that this difference in binding cannot be explained neither by dissimilar efficiencies in immunoprecipitating Flag-Rep68, nor by diverse expression levels of the transgenic constructs in the different samples. From Figure 3.13b it is also possible to appreciate, by immunoblotting against Ku70, that the micrococcal nuclease treatment of the immunocomplexes completely abolished Flag-Rep68 binding to Ku70, while the binding of the former to ANP32B was resistant to such treatment. This observation suggests that the interaction between Rep68 and ANP32B is independent of the presence of DNA.

It is interesting to observe that ANP32B migrates slightly faster than ANP32A, despite the two proteins being 251 and 249 aa long, respectively. Nonetheless, this peculiar behavior of ANP proteins in migration on a SDS-PAGE gel is well in agreement with previous reports in the literature (Brennan et al., 2000), (Jiang et al., 2003) and can be explained by considering that ANP32 proteins are heavily post-translationally modified proteins (Hong et al., 2004; Mencinger et al., 1998; Yu et al., 2004).

We then sought to confirm the results presented in Figure 3.13 by repeating the same experiments, but this time immunoprecipitating cell lysates with an antibody against HA and immunoblotting the precipitated proteins with an anti-Flag antibody. The results of this experiment (Fig. 3.14) are in perfect agreement with the previous observations. In fact, also in this case, Flag-Rep68 was immunoprecipitated by the HA antibody only in the presence of ANP32B (Fig. 3.14, lane six), but not in the presence of ANP32A (Fig. 3.14, lane five). Again, this

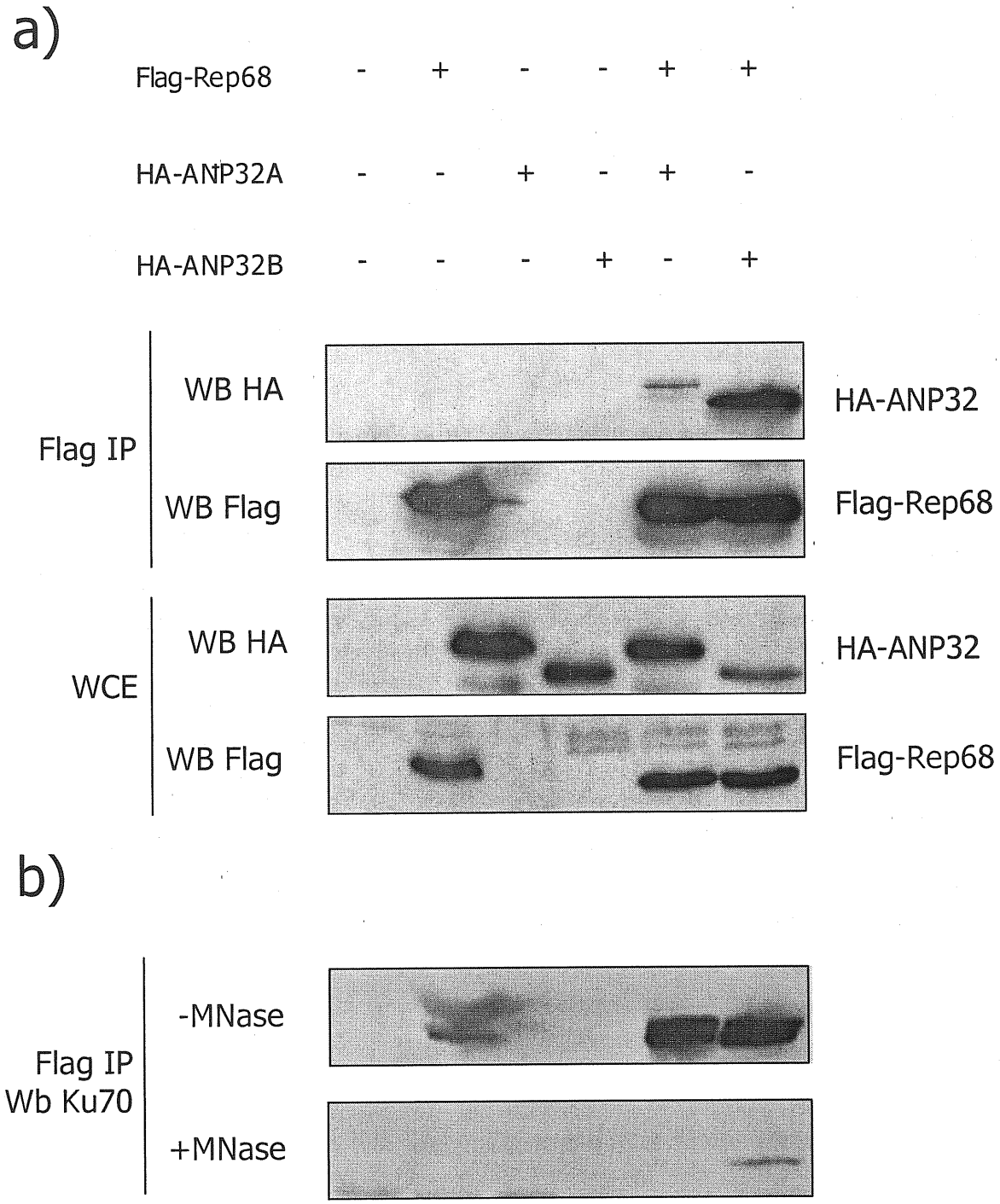


Fig. 3.13) Flag-Rep68 binds ANP32B but not ANP32A in a DNA independent manner. a) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with M2 anti-Flag agarose beads. Immunoprecipitates were treated with micrococcal nuclease, run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure. b) The same immunoprecipitates were western blotted against KU70 to show that the micrococcal nuclease treatment was effective. WCE: whole cell extract. MNase: micrococcal nuclease.

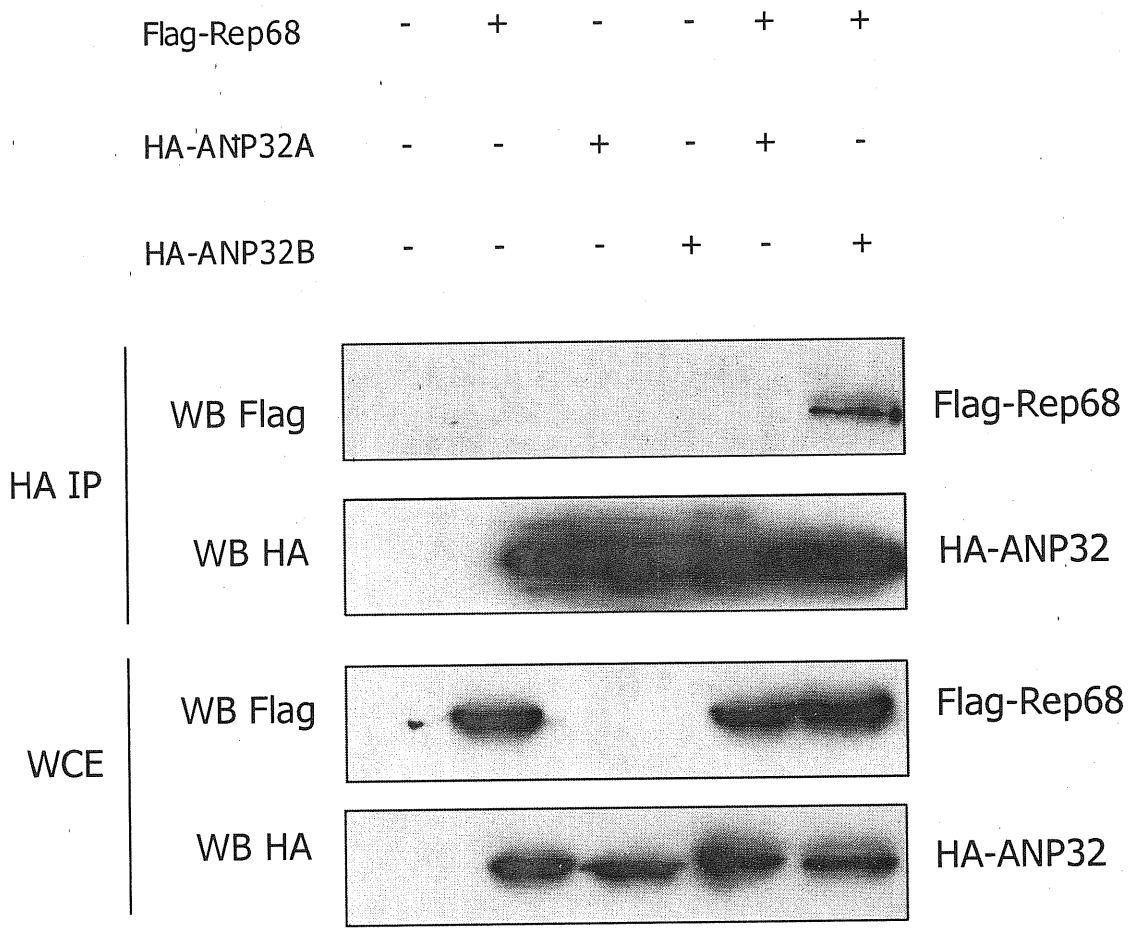


Fig. 3.14) Flag-Rep68 binds ANP32B but not ANP32A *in vivo*. HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with 3F10 anti-HA agarose beads. Immunoprecipitates were loaded and run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure. WCE: whole cell extract.

difference was neither due to different expression levels of the transgenes nor to differences in HA immunoprecipitation efficiency, as shown by the immunoblots against HA and Flag on cell lysates and immunocomplexes respectively.

To test whether the binding of ANP32B to Rep68 could be reproduced *in vitro*, we first produced recombinant HisRep68 in E.*coli* and purified it on a nickel column. On the bound protein, we applied *in vitro* transcribed and translated ANP32A or ANP32B radiolabelled with ^{35}S methionine. After several washes, the proteins bound to Rep68 were eluted with Laemli buffer and the products were run on a 12% SDS-PAGE gel (Fig. 3.15). Radiolabelled proteins retained on the column were quantified and measured as a percentage of the initial total input (Fig. 3.15a, lanes one and two). The Ni-Nta column alone did not display any unspecific binding neither to ANP32A nor to ANP32B (Fig. 3.15a, lanes three and four). Instead, we could demonstrate that HisRep68 was able to retain 25% of radiolabelled ANP32B (Fig. 3.15a, lane six). In contrast, only 2.5% of the initial ANP32A was retained by HisRep68 (Fig. 3.15a, lane five), a clear indication that that Rep discriminates between the two different ANP family members. As a control, the Coomassie stained gel shown in Figure 3.15b indicates that the quantity of HisRep68 used in this assay was the same in both samples.

Taken together, the *in vitro* results presented so far confirm the experimental observations that we obtained with the co-immunoprecipitations *in vivo* (Fig. 3.13 and Fig. 3.14).

3.10 The N-terminal of Rep68 is necessary for its interaction with ANP32B.

Once the selectivity of the interaction between Rep68 and ANP32B was confirmed, we wanted to define which domain of Rep was involved in the binding. Upon productive AAV-2 infection, four different isoforms of Rep are produced through the use of alternative start sites and alternative splicing (See Introduction chapter). As mentioned previously, Rep40 corresponds to residues 225-526 of Rep68 and it is the shortest natural isoform produced by AAV-2 (Figure 3.16b). As

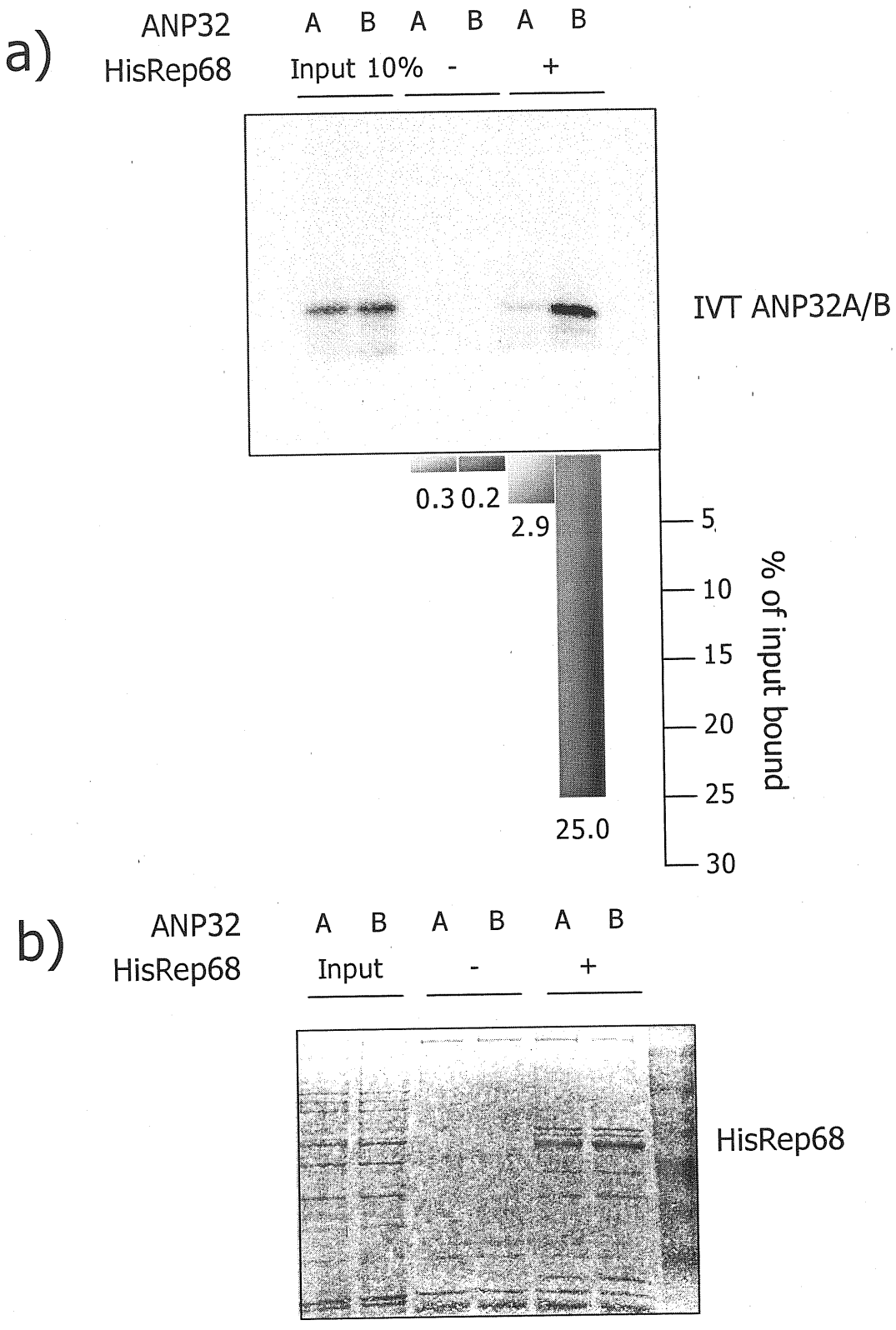


Fig. 3.15) Rep68 binds ANP32B but not ANP32A *in vitro*. a) His-Rep68 bound on Ni-Nta agarose beads was incubated with *in vitro* transcribed and translated ^{35}S methionine labeled ANP32 proteins. The level of bound ANP32 proteins was measured as the quantity of radioactivity that was retained on the His-Rep68 beads and expressed as percentage of the input. b) Coomassie Stain of the gel.

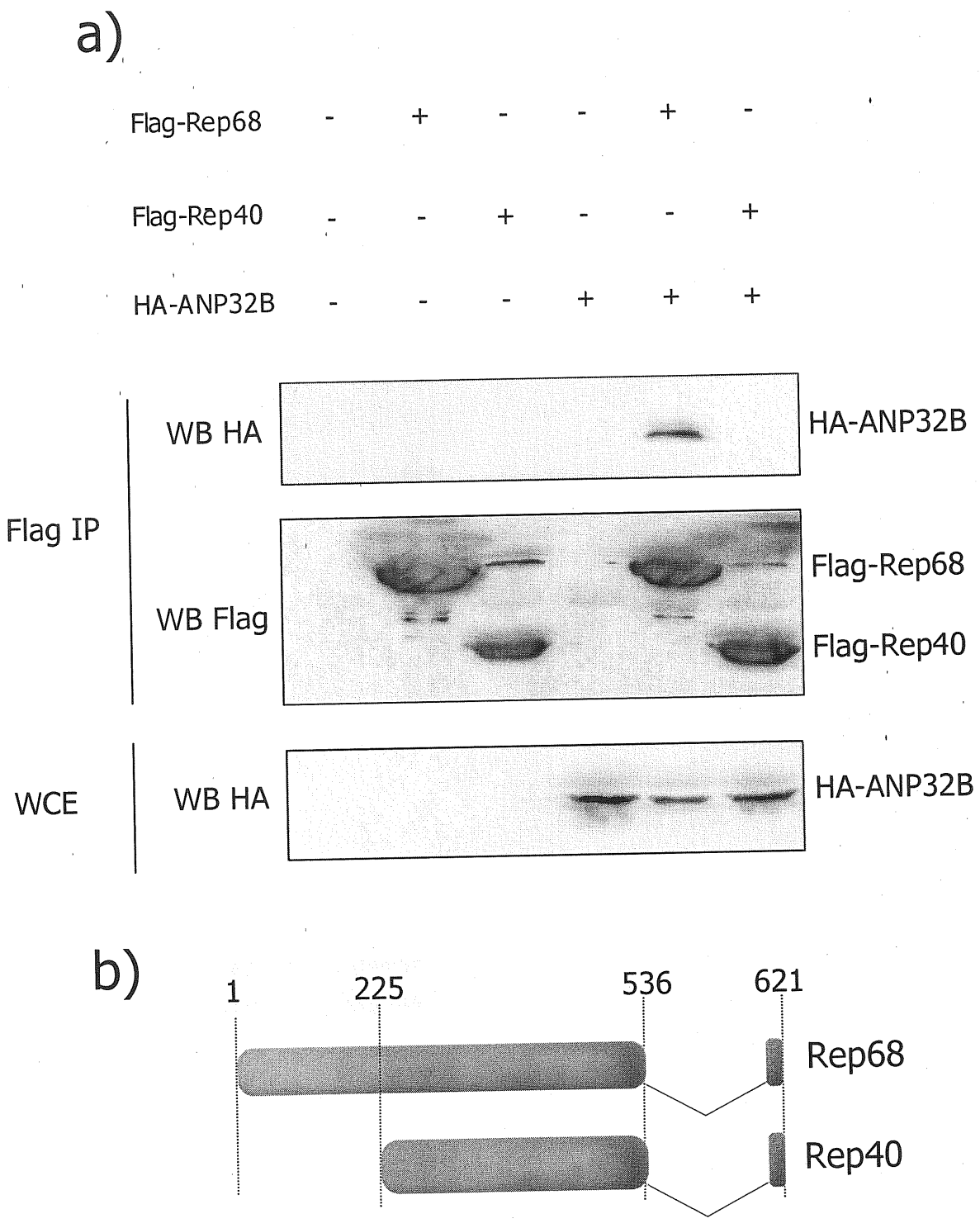


Fig. 3.16) Flag-Rep40 does not bind ANP32B. a) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared and immunoprecipitated with M2 anti-Flag agarose beads. Immunoprecipitates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure. b) Schematic representation of Rep68 and Rep40. Aa positions are indicated at the top. WCE: whole cell extract. Mnase: micrococcal nuclease.

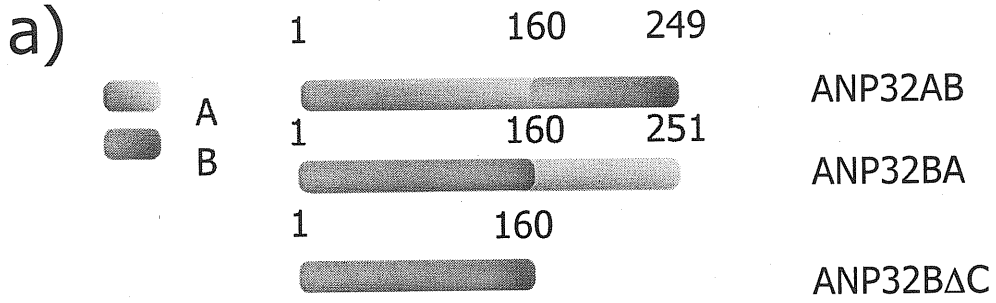
shown in Figure 3.16a, when we used this N-terminally truncated isoform we were not able to immunoprecipitate ANP32B (Fig. 3.16, lane six), whereas, in the same experimental conditions, Flag-Rep68 did indeed bind ANP32B (Fig. 3.16, lane five). This result indicates that the first 224 N-terminal residues of Rep are necessary for the physical interaction. This domain is endowed with DNA binding and endonuclease activities and it is shared only by the two largest isoforms, i.e. 78 and 68 (Im and Muzyczka, 1990).

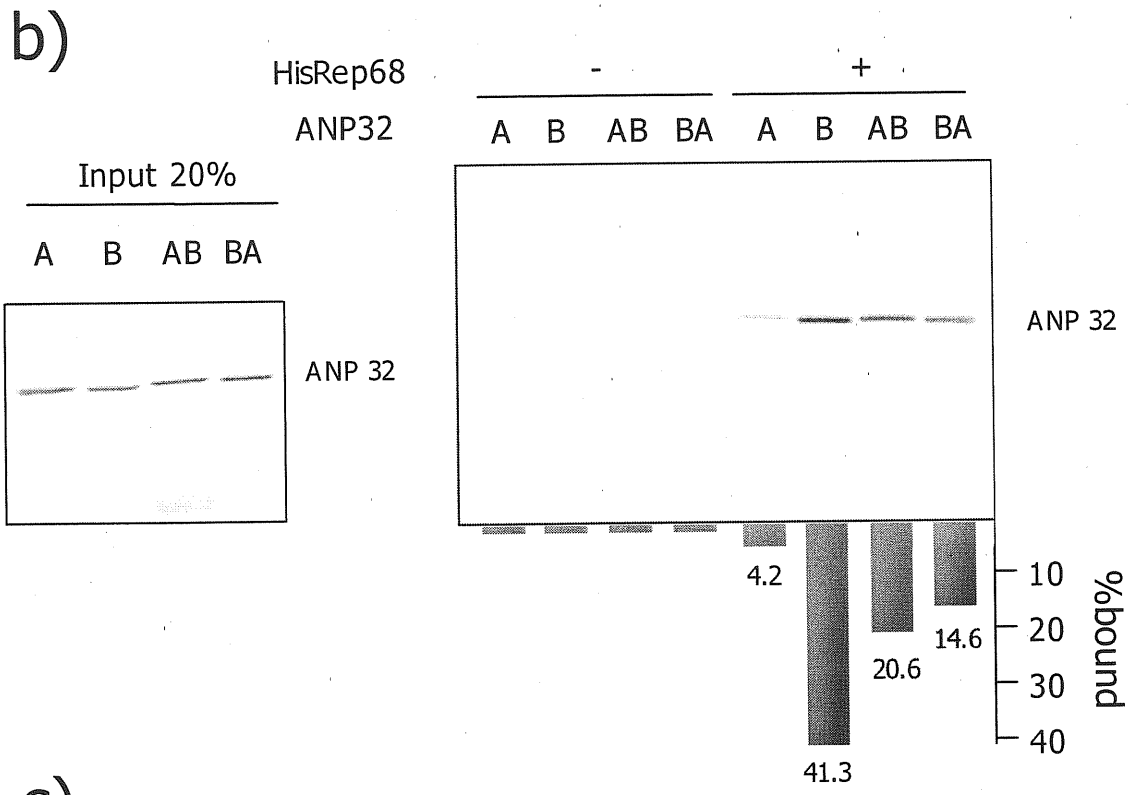
In summary, this set of experiments (Fig. 3.13, 3.14,3.15 and 3.16) confirms the results obtained by Mass Spectrometry analysis (Fig. 3.10 and Tab. 3.1). In fact, we were able to demonstrate that: a) the *in vivo* and *in vitro* binding to Rep68 is specific to ANP32B and not to ANP32A, even though the two of them share extensive sequence identity; b) this interaction is DNA independent, since it is insensitive to micrococcal nuclease; and c) the first 224 aa of Rep are necessary for such interaction.

3.11 Domains of ANP32B important for the interaction with Rep68.

In a further attempt to determine which region of ANP32B contacted Rep68, we constructed two ANP swap mutant constructs that were designated as ANP32AB and ANP32BA, respectively. These two expression plasmids produced chimeric proteins that contained the first 160 residues of ANP32A fused to the last 90 residues of ANP32B, and vice versa (Figure 3.17a).

When used in the *in vitro* binding assays, 20.6% of ANP32AB and 14.6% of ANP32BA remained bound to HisRep68 (Fig. 3.17b, lanes seven and eight). These values were roughly one half of the binding value displayed by wtANP32B, which was 41.3% (Fig. 3.17b, lane six). On the other hand, in the same experimental conditions wtANP32A did not bind HisRep68 (Fig. 3.17b, lane five). These findings suggest that both the N-terminal region and the C-terminal regions of ANP32B are involved in the binding to Rep68.


a)
1
160
249
A
B
ANP32AB
1
160
251
ANP32BA
1
160
ANP32BΔC


b)
HisRep68
-
+
ANP32
A B AB BA A B AB BA
Input 20%
A B AB BA
ANP 32
ANP 32
4.2
41.3
20.6
14.6
10
20
30
40
%bound

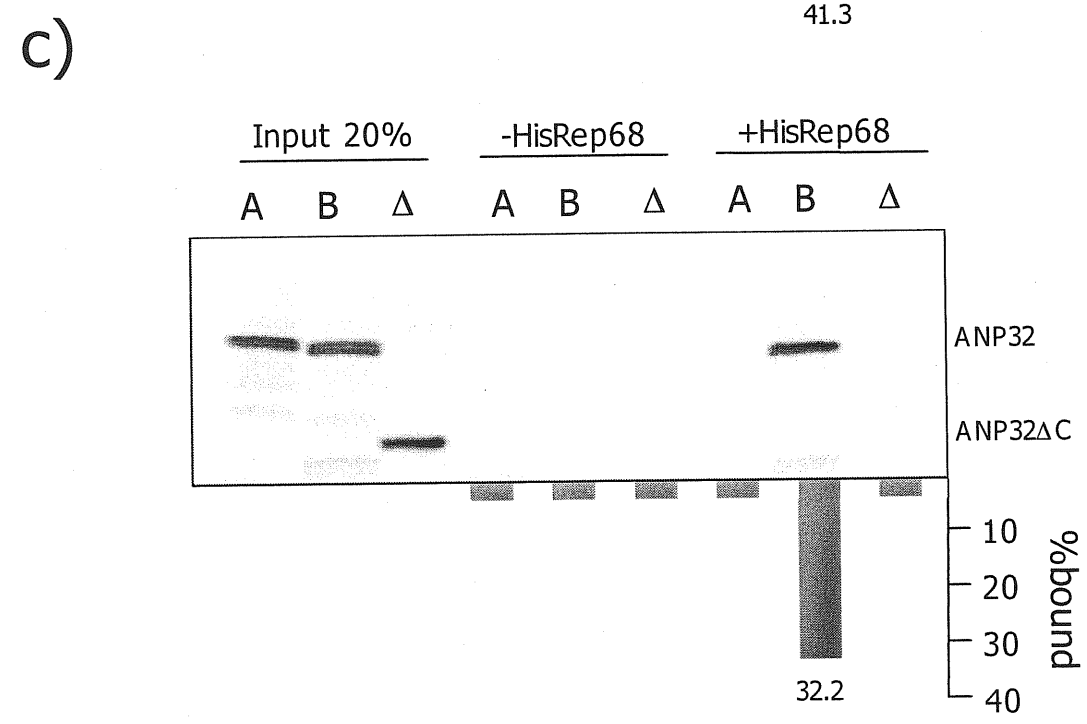

c)
Input 20%
-HisRep68
+HisRep68
A B Δ A B Δ A B Δ
ANP32
ANP32ΔC
32.2
10
20
30
40
%bound

Fig. 3.17) The C-terminus of ANP32B is necessary for the interaction with Rep68. a) Schematic representation of the ANP32 mutants used in this figure. The numbers over the boxes indicate the aa position where domains were either swapped or deleted. b) His-Rep68 bound on Ni-Nta agarose beads was incubated with *in vitro* transcribed and translated ^{35}S methionine labeled swap mutants of ANP32 proteins. The level of bound ANP32 proteins was measured as the quantity of radioactivity that was retained on the His-Rep68 beads and expressed as percentage of the input. c) Same as b) but a C-terminal deletion mutant of ANP32B (ANP32BΔC) was used instead.

However, ANP32B ΔC (Fig. 3.17a), a deletion mutant of the C-terminal domain of ANP32B, did not bind HisRep68 (Fig 3.17c, lane nine), indicating that a major contribution in the physical interaction is due to this region of ANP32B. Taken together these results indicate that: a) the C-terminal domain of ANP32B is necessary for the interaction; and b) discrimination between ANP32A and ANP32B arises as a more complex recognition of other regions, present in the N-terminus of the protein. Clarification of this aspect will clearly require the resolution of the molecular structure of ANP32 proteins.

3.12 Functional role of ANP32 proteins in increasing AAV replication.

Rep68, and Rep78, are fundamental for AAV replication and integration. In particular, the region involved in ANP32 binding (the first 224 residues) is fundamental for Rep to promote these activities *in vivo*, since Rep40 and Rep52 still retain helicase activity but are not competent in replication or integration. Considering the biochemical evidences presented in the previous paragraphs, we thought that ANP32B might have a role in the regulation of Rep68 functions. To test such hypothesis we decided to set up an *in vivo* AAV replication assay. We took advantage of the Adenovirus-free helper system that employs the previously described plasmid pDG (Grimm et al., 1998). Briefly, this plasmid, which is routinely used for the production of recombinant AAV vectors, encodes for the AAV Rep and Cap genes, for the Ad VA1 RNA, and for the Ad E2A and E4 genes. In this way all the functions needed for AAV replication are supplemented in trans by a single plasmid, whereas the substrate to be replicated is a plasmid, in our case pTR-UF5, that contains a GFP and a neomicine-resistance expression cassettes flanked by the two AAV ITR.

Low molecular DNA was extracted by a modified Hirt procedure from HEK 293 cells transfected with a combination of these plasmids and with the expression vectors for HA-ANP32A and HA-ANP32B (See Material and Methods chapter). Samples were digested with DpnI to remove input plasmids, run on a 0.7% agarose gel,

Southern blotted on a nylon membrane and then replication products were visualized with a specific ^{32}P radiolabelled DNA probe.

As expected, in the absence of the replication functions provided by the pDG plasmid, no replication products could be visualized by autoradiography (Fig 3.18a, lane one). The two ANP32 proteins alone did not support AAV replication in the absence of pDG (Fig 3.18a, lanes two and three). Instead, when pDG was cotransfected together with pUF5 two main bands, and a fainter, faster migrating band appeared (Fig 3.18a, lane four). Based in their estimated molecular weight, these bands correspond to the monomeric, dimeric and trimeric replication intermediates of the AAV genome named RF1,RF2 and RF3 (Fig 3.18). When either ANP32A or ANP32B were co-transfected together with pTR-UF5 and pDG, the quantity of rAAV replication products increased consistently (Fig 3.18a, lanes five and six).

By quantifying the band intensity with Phosphoimager analisys, we could observe a reproducible 2.5 fold increase in the pDG+ANP32 transfected samples compared to the sample that was transfected by pDG alone (Fig. 3.18b). From this result, and considering that ANP co-transfection did not change the total amount of the different Rep isoforms in the samples transfected with pDG (Fig. 3.18c), we concluded that both ANP proteins can increase efficiency by which Rep replicates rAAV genomes.

Noticeably, the *in vitro* and *in vivo* binding data predicted that only ANP32B had an effect on AAV DNA replication. Instead, the increase was virtually identical when using either ANP32A or ANP32B. This point will be extensively discussed later on.

3.13 Development of siRNA against ANP32A and ANP32B.

Having observed an increase in AAV replication upon ANP32 over-expression (Fig 3.18), we sought to determine whether the ANP32 proteins are indeed involved to AAV replication *in vivo*. We then chose to take advantage of the siRNA technology to knock-down ANP32 protein expression.

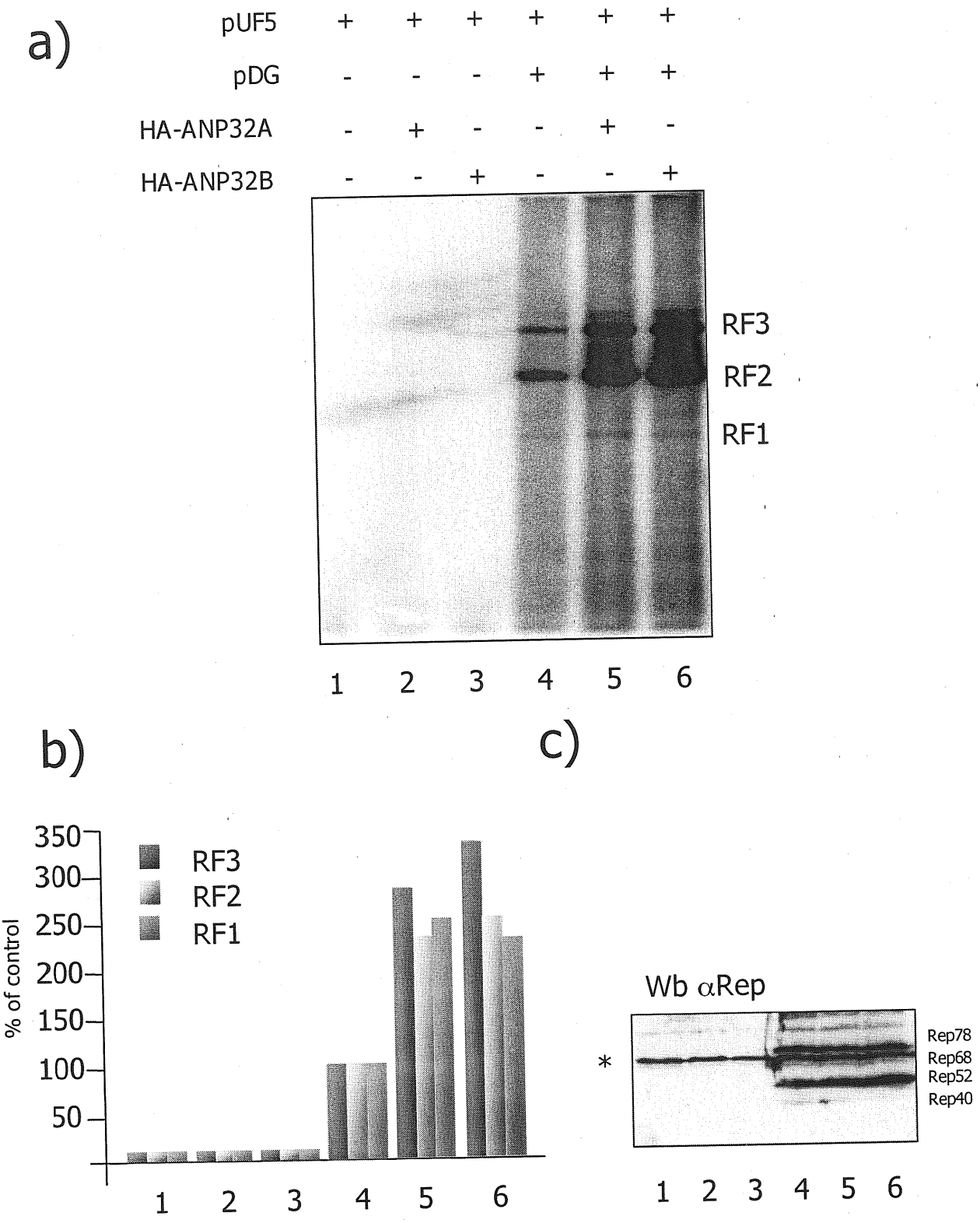


Fig. 3.18) ANP32 proteins increase AAV replication efficiency. a) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs. Hirt extracted DNA was digested with DpnI, run on a 0.7% TAE agarose gel, and transferred to a nylon membrane. Specific rAAV replication bands were identified by using a 1kb fragment of XhoI digested pTR-UF5 as a probe. Replication intermediates RF1, RF2 and RF3 are indicated. b) The normalized intensity (Number 4: 100%) of the bands present in a) was reported in this histogram. c) An aliquot of cell lysates from the same experiment was loaded on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and blotted with an antibody against Rep. The asterisk denotes an unspecific band.

Target sequnce selection is a fundamental process in order to get efficient and specific siRNA gene silencing. In order to achieve these goals we followed the criteria recently established by Reynolds *et al.* (Reynolds et al., 2004). Briefly, the parameters for target sequence selection indicated by these authors take in consideration thermodynamic and primary sequence parameters of the target sequence to enhance the efficiency of silencing by siRNA. After careful screening of candidate sequences present in the cDNAs of ANP32A and ANP32B, we chose one of them for each gene (Fig. 3.19a). From a brief analysis of the siRNAs that we chose, it is possible to appreciate that the two target sequences differ from each other just in three nucleotide positions (Boxed in red). According to previous reports, a difference of one single nucleotide between two different siRNAs is sufficient to achieve gene silencing specificity (Elbashir et al., 2001). Proper oligonucleotides corresponding to the sequences shown in Fig. 3.19a were synthesized and cloned into the RNA PolIII expression plasmid pSUPER for expression of double stranded siRNA oligonucleotides in cell culture (Brummelkamp et al., 2002). To test for siRNA specificity and functionality we set up a transfection experiment in HEK293 cells using both GFP, HA-ANP32 and HA-ANP32B expression vectors and pSuper plasmids against the same proteins. As a control, pSuperGFP transfection had no effect on HA-ANP32A or HA-ANP32B protein levels, whereas it effectively silenced GFP expression (Fig 3.19b, lanes one and two). pSuperANP32A inhibited HA-ANP32A protein levels down to background (Fig 3.19b, lane three), but had no effect on either GFP or HA-ANP32B expression (Fig 3.19b, lanes three and four). At the same time, pSuperANP32B completely abolished the levels of HA-ANP32B (Fig 3.19b, lane six) without influencing HA-ANP32A or GFP levels (Fig 3.19b, lanes five and six). Collectively, these results indicate that the two siRNAs are specific for their target sequence, that they show no effect on the related isoform, and that they both are highly effective in promoting degradation of their respective genes.

a)

ANP32A	673	GAAGAGGAGTTTGATGAAGAA	693
ANP32B	559	GAAGAAGAGCTTGGTGAAGAA	579

b)

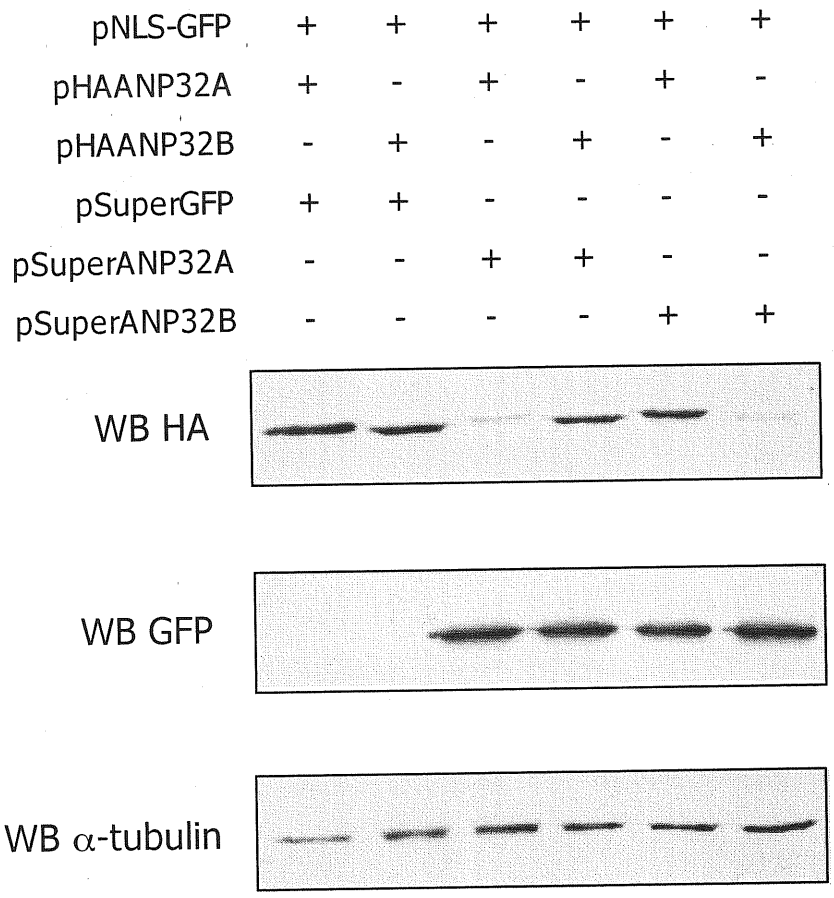


Fig. 3.19) pSuperANP32A and pSuperANP32B specificity. a) The target sequences in ANP32A and ANP32B mRNA are reported. The beginning the end of the target sequences, relative to their position in the mRNA, are indicated. Single nucleotide differences between ANP32A and ANP32B target sequences are boxed in red. b) HEK 293 cells were transfected with the indicated combinations of plasmids. After 36 hrs cell lysates were prepared, run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The latter was probed with specific antibodies as indicated in the figure.

3.14 ANP32B is involved in AAV replication.

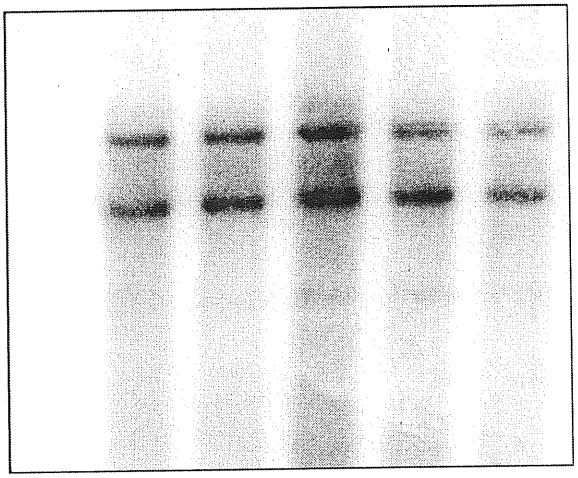
To test whether ANP32A and ANP32B are involved in AAV replication, pSuperANP32A and pSuperANP32B were then transfected together with pTR-UF5 and pDG, and AAV replication assays were carried out essentially as outlined in the Materials and Methods chapter. The results of these experiments are collectively presented in Figure 3.20. In particular, the autoradiogram of a representative experiment is presented in Figure 3.20a.

As expected, no bands were present in the absence of the AAV and Adenovirus replication factors encoded by pDG (Fig. 3.21a, lane one), whereas the AAV replication intermediates RF2 and RF3 appeared upon co-transfection of pDG (Fig. 3.20a, lane two). The intensity of these bands did not change when pSuperGFP, the control vector that has no influence on ANP32 levels, was added to the transfection mix (Fig. 3.20a, lane three). The same held true for pSuperANP32A (Fig. 3.20a, lane four), whereas in the case of pSuperANP32B alone (Fig. 3.20a, lane five), or in the case of a combination of pSuperANP32B together with pSuperANP32A (Fig. 3.20a, lane six) we could reproducibly observe a reduction in the intensity of the RF3 band. As a control, the immunoblot against Rep shown in Figure 3.20c clearly indicates that the pSuper plasmids had no effect on expression levels of the Rep proteins.

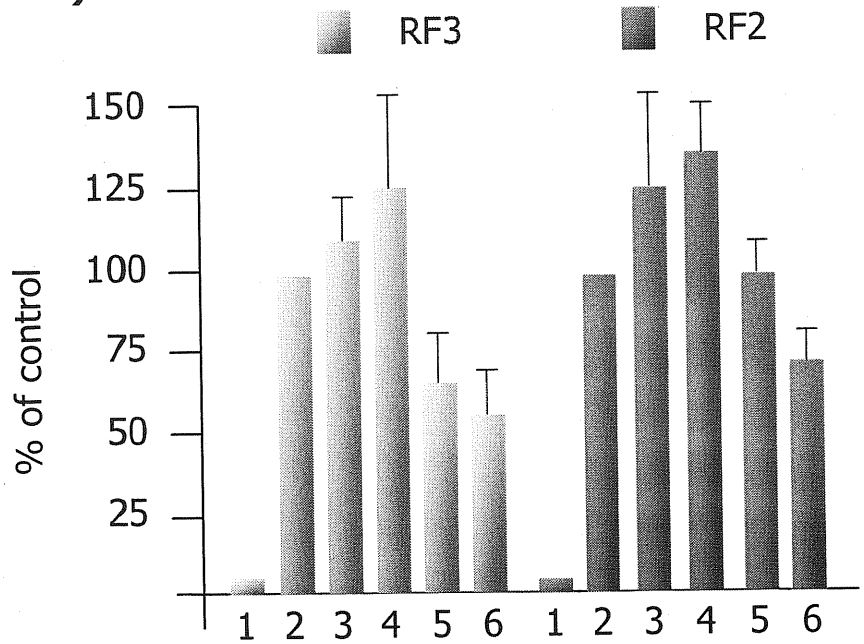
The AAV replication assay in the presence of different siRNAs was independently repeated three times, the intensities of RF3 bands were quantified by Phopsoimager analisys, and their average value was plotted in the histogram shown in Figure 3.120b. Importantly, the siRNA against ANP32B, but not against ANP32A, resulted in almost 50% decrease in AAV replication efficiency. Thus, in agreement with the biochemical data presented in the first part of this work, we were able to demonstrate that only ANP32B is involved in AAV replication. The apparent change in the ratio between RF3 and RF2 upon silencing of ANP32B could be repeated two out of three times and thus more experiments will be needed to confirm the significance of this observation.

a)

pUF5	+	+	+	+	+	+
pDG	-	+	+	+	+	+
pSuperGFP	-	-	+	-	-	-
pSuperANP32A	-	-	-	+	-	+
pSuperANP32B	-	-	-	-	+	+



b)



c)

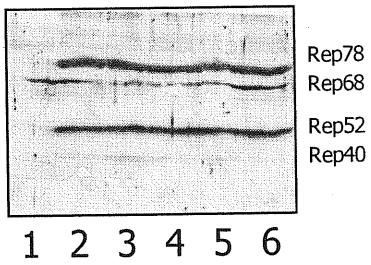


Fig. 3.20) Silencing of ANP32B decreases AAV replication efficiency. a) HEK 293 cells were transfected with the indicated combination of plasmids. After 36 hrs. Hirt extracted DNA was digested with DpnI, run on a 0.7% TAE agarose gel, and transferred to a nylon membrane. Specific AAV replication bands were identified by using a 1kb fragment of XhoI digested pTR-UF5 as a probe. Replication intermediates RF1, RF2 and RF3 are indicated. b) The experiment was repeated three times and the mean normalized intensity (Number 2: 100%) of the bands present in a) was reported in this histogram. c) An aliquot of cell lysates from a) was loaded on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and blotted with an antibody against Rep.

3.15 ANP proteins enhance the production of the pTR-UF5 viral vector.

Since the AAV replication levels were enhanced by over-expressing ANP32A or ANP32B, we investigated whether they can also increase the viral titers of rAAV vectors. Small-scale vector productions experiments (See the Material and Methods chapter) were set up in order to verify this hypothesis. HEK 293 cells were transfected in duplicate with pTR-UF5 and pDG (A 3:1 ratio between the two plasmids was used as published (Grimm et al., 1998)) plus or minus plasmids expressing HA-ANP32A or HA-ANP32B. After 24 hrs, virus particles were released from producing cells by freeze/thaw and precipitated in ammonium sulphate. The pellet was resuspended and extensively dialyzed in PBS to remove salts, and total protein levels were quantified for each preparation and accordingly normalized. Samples were first digested with DNAse I to remove non-encapsidated genomes, and then capsid proteins were removed by proteinase K digestion. Finally, viral titers were determined by competitive quantitative PCR.

In keeping with the previous findings, we were able to obtain an increase in viral titers when either HA-ANP32A or HA-ANP32B were transfected together with pTR-UF5 and pDG (Fig. 3.21a). When the intensity of the DNA bands was measured by densitometry (Fig 3.21b), we could observe a ~2.5 fold increase in the vector titer when HA-ANP32A or HA-ANP32B were included in the transfection mix. In these conditions could we not observe any further increase by the concomitant co-transfection of the HA-ANP proteins together (data not shown). This experiment was repeated three times with similar results.

The 2.5 fold increase in viral titer shown in Figure 3.21 does not only suggest that ANP proteins can be used as a biotechnological tool to improve vector production, but is an independent confirmation of the conclusions obtained from the quantification of AAV replication experiments (Fig 3.19b).

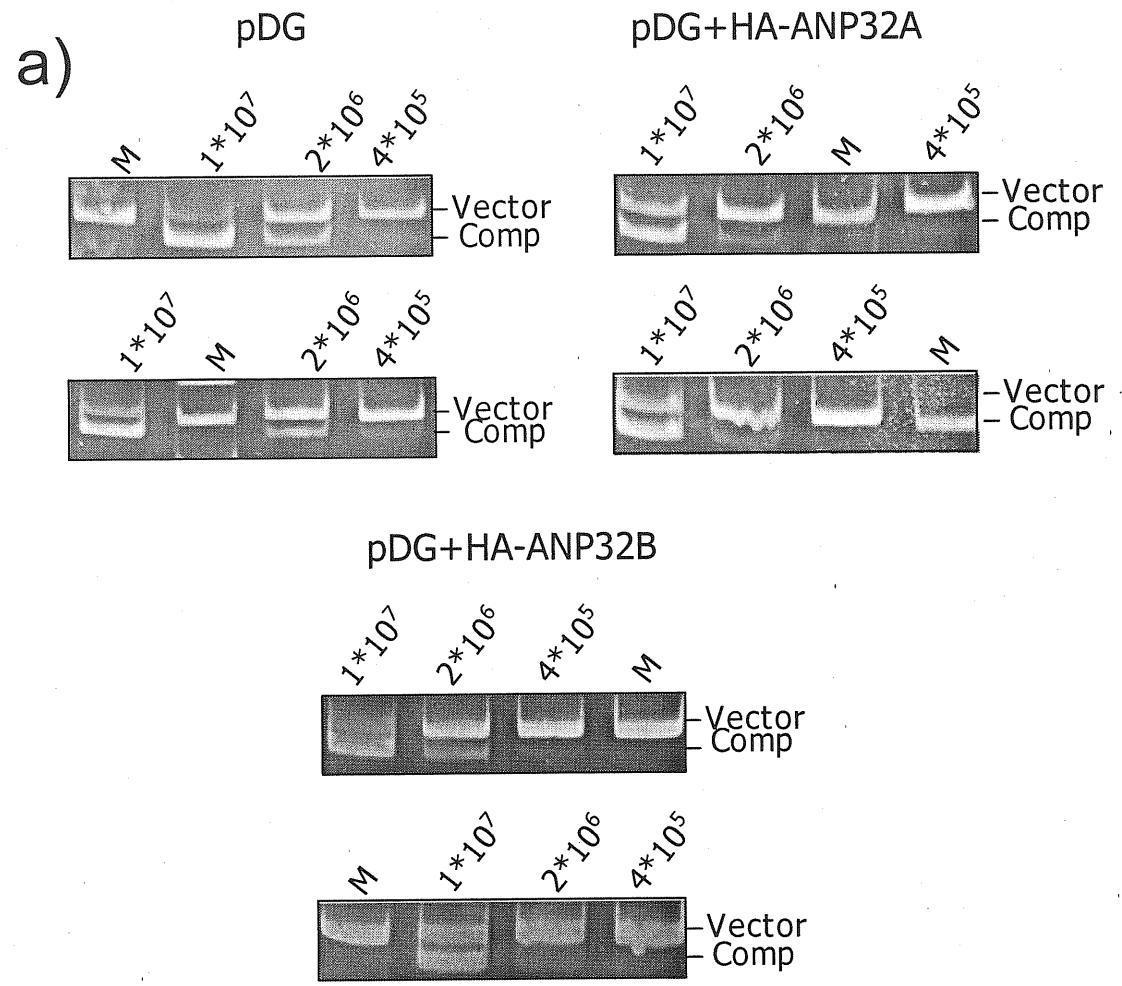


Fig. 3.21) ANP proteins increase AAV vector production. a) HEK 293 cells were transfected in duplicate with pTR-UF5 and the indicated combination of plasmids. After 36 hrs. AAV vectors were prepared as described in the Materials and Methods chapter. Encapsidated AAV genomes were quantified by competitive PCR. The position of the amplified bands corresponding to the competitor and to the rAAV genome is indicated. On the top of the panels the concentration of the competitor is indicated as well as the DNA marker lane (M). Each panel represent one of the two duplicates. b) The band intensity in a) was measured by densitometry and the number of rAAV genomes per ml was plotted. 1: pDG, 2: pDG+HA-ANP32A, 3: pDG+HA-ANP32B.

Chapter 4: Discussion.

AAV molecular biology is interesting from many points of view: AAV integrates at a specific locus in chromosome 19, its replication is dependent on Adenovirus co-infection or DNA damage and it blocks cellular DNA replication, cell cycle progression and RNA transcription of several cellular and viral genes; moreover, all these events need the presence of the AAV Rep proteins. In this respect, it is evident that the study of AAV and the Rep proteins is important not only for an improved knowledge of the virus itself, but it can also give valuable new insight into these cellular pathways. Finally, information on basic AAV biology is of great importance for the design and production of improved rAAV vectors for gene therapy.

In this work we present experimental evidences showing that molecular interactions of Rep with host cell proteins regulate cellular transcription and AAV replication. For the sake of clarity these two topics will be discussed in two separate sections.

4.1 Rep inhibition of p300-mediated transcription and the involvement of AMF-1/Gps2.

In a previous study it has been demonstrated that AAV Rep inhibits HPV16 E2 mediated transcription by interacting with the latter and by disrupting its interaction with the transcriptional co-activator, histone acetyltranferase p300 (Marcello et al., 2000). Since p300 is a transcription cofactor used by many other cellular and viral transcription factors, we sought to extend the model proposed for HPV16 E2 to other cellular and transcriptional activators. HIV-1 Tat and hE2F1 were chosen as candidate proteins because they are both p300-dependent transcriptional factors (Marzio et al., 1998; Marzio et al., 2000). Moreover, previous work showed that Rep is capable of inhibiting transcription from the HIV-LTR promoter (Batchu and Hermonat, 1995; Horer et al., 1995; Kokorina et al.,

1998). It was also previously demonstrated that Rep binds E2F1 and stabilize it in complex with Rb, thus inhibiting E2F1-mediated transcription and cellular proliferation (Batchu et al., 2001). Accordingly, Fig. 3.1 clearly shows that Rep is capable of inhibiting the transcriptional activity of both these activators. We had previously ruled out any unspecific effect of Rep in these kind of transcriptional assays, either on transcription or on cell viability, by showing that in our experimental conditions Rep68 did not consistently influenced the activity of a constitutive CMV-driven promoter (Marcello et al., 2000). Our results are well in agreement with previously published observations (Batchu et al., 2001; Horer et al., 1995). However, it has been proposed that Rep acts through binding to the LTR promoter itself (Batchu and Hermonat, 1995). In this respect, it is worth noting that the synthetic E2F1 reporter we used does not contain an RBS in the proximity of the transcription start site (Fig. 1b). Therefore we are keen to think that Rep inhibitory effect in our experimental system is due to protein-protein interactions rather than to DNA binding by Rep. This notion is strengthened by the data presented in Fig. 3.2, in which Rep is able to disrupt the interactions between HIV-1 Tat or hE2F1 with p300. In the case of HPV16 E2, it was possible to demonstrate that the Rep domain involved in this interaction could be limited to aa 225-526, corresponding to the natural isoform Rep40 (Marcello et al., 2000). The crystal structure of AAV-5 Rep40 has been recently solved, and by 3D model comparison it was possible to demonstrate that this domain belongs to the SF3 AAA^+ ATPase family (James et al., 2003) (See Chapter 1). This family of cellular proteins is involved in the active remodeling of DNA and protein complexes at the expense of ATP. Given that Rep40 is able to disrupt physical interactions among transcriptional protein complexes, and considered that AAA^+ proteins are not only DNA helicases but also have the capability of remodeling the quaternary structure of protein complexes (Lee et al., 2004), its tempting to speculate that this Rep activity is not a passive phenomenon, but rather is an active process driven by ATP consumption. Further experiments with ATPase/Helicase mutants in transcriptional assays and *in vitro* binding assays will be needed to prove this hypothesis.

The observation that Rep targets the interaction of so many different cellular and viral targets with p300 (Which does not directly bind Rep, data not shown) can be

explained by assuming the presence of a common bridging factor in these protein complexes, which in turn is directly targeted by Rep to exert transcriptional inhibition.

Previous work by the Androphy laboratory showed that AMF-1 is involved in the regulation of BPV E2 transcription (Activation domain Modulating Factor 1) by directly binding the N-terminal activation domain of this viral transcription factor (Breiding et al., 1997), and by helping in the recruitment of the Histone Acetyltransferase (HAT) co-activator p300 (Peng et al., 2000). In line with these observations, it was also shown that AMF-1 modulates p53-mediated transcription (Peng et al., 2001). Hence, it might appear that AMF-1 has just a positive role on transcriptional regulation. However, more recently, the Roeder laboratory has shown that AMF-1 is also an integral component of the N-CoR/HDAC3 repressive complex, which is endowed with histone deacetylase activity and is consequently involved in the repression of transcription when tethered to DNA (Zhang et al., 2002). The most likely interpretation of these apparently contradictory evidences is that AMF-1 can participate in different protein complexes having opposite functions, and that the equilibrium between activation and repression can be shifted by stimuli of various origin. Human AMF-1/Gps2 was originally described as a suppressor of G-protein pathways (Hence the name Gps2) in yeast and of the Ras pathway in human cells (Spain et al., 1996). In a subsequent report AMF-1 was subsequently identified as a protein partner for HTLV-1 Tax in a two-hybrid assay (Jin et al., 1997). It was also shown that AMF-1 is able to bind Tax and to inhibit the signal transduction pathway that starts from TNF-α and converge on JNK-1. Aside from playing an important role at the level of transcription, AMF-1 was also involved in the regulation of transduction of cellular signals from the cell surface to the nucleus.

Intriguingly, AMF-1 binds HIV-1 Tat and E2F1 *in vitro* and *in vivo* (Fig. 3.3 and 3.4) and has co-activator functions for these factors in transcriptional assays (Fig 3.5). These data are in line with what was previously demonstrated for the interaction between AMF-1 and HPV16E2 (Breiding et al., 1997) and between AMF-1 and p53 (Peng et al., 2001), which resulted in transcriptional co-activation of these two transcription factors.

Once that the physical interaction with HIV-1 Tat and hE2F1 was demonstrated for AMF-1, we then sought to see whether this cellular co-factor can also be a protein partner for Rep. This hypothesis was demonstrated *in vivo* and *in vitro* (Fig. 3.6a and 3.6b). Interestingly, Rep 40 alone, corresponding to the C-terminus of Rep68, was capable of binding with AMF-1 *in vitro* (Fig. 3.6b). This last evidence fits well with the model proposed for the effect of Rep on HPV16, in which over expressed Rep40 was able to repress transcription and replication (Marcello et al., 2000). Thus AMF-1 might well be the physical mediator of the Rep disruption between p300 and the transcriptional co-activators ((Marcello et al., 2000), Figure 3.2a and 3.2b). Data presented in Figure 3.7a and 3.7b support this model: in fact, Rep dislocates AMF-1 from p300, but not from HPV16 E2. This last experimental evidence points to two important facts: first, the effect of Rep on protein binding is not generalized, but instead is directed toward specific protein-protein contacts. Second, even in the presence of Rep HPV16 E2 remains bound to AMF-1. Given that the latter has also been found to be part of the N-CoR protein complex that has transcriptional inhibitory activity (Zhang et al., 2002), we propose a working model in which Rep not only inhibits the formation of a transcriptional active complex by impeding the access of p300 to the reaction, but also shifts AMF-1 toward the formation of a repressive complex that also includes HDAC3, N-CoR and TBL1 (Fig 4.1). This double-faceted role for AMF-1 as a switch between activating (p300-containing) and repressive (N-CoR) complexes is not entirely new. In fact, TBL1, another integral component of the N-CoR complex, has been shown to remain bound to the Estrogen Receptor (the transcription factor in this experimental system) and to swing between the above mentioned protein complexes depending on different active/inactive states (Perissi et al., 2004). Figure 3.8a and 3.8b partially support this hypothesis by showing that that Rep is capable of recruiting HDAC3 in co-immunoprecipitations *in vivo* and in HisRep68 pull-down assays of HeLa Nuclear extracts *in vitro*.

Further experiments will be directed to assess the actual presence of N-CoR in Rep containing complexes.

Altogether, the data presented in this first part of the thesis explain, at a molecular level, the events that lead to the Rep mediated repression of the transcriptional

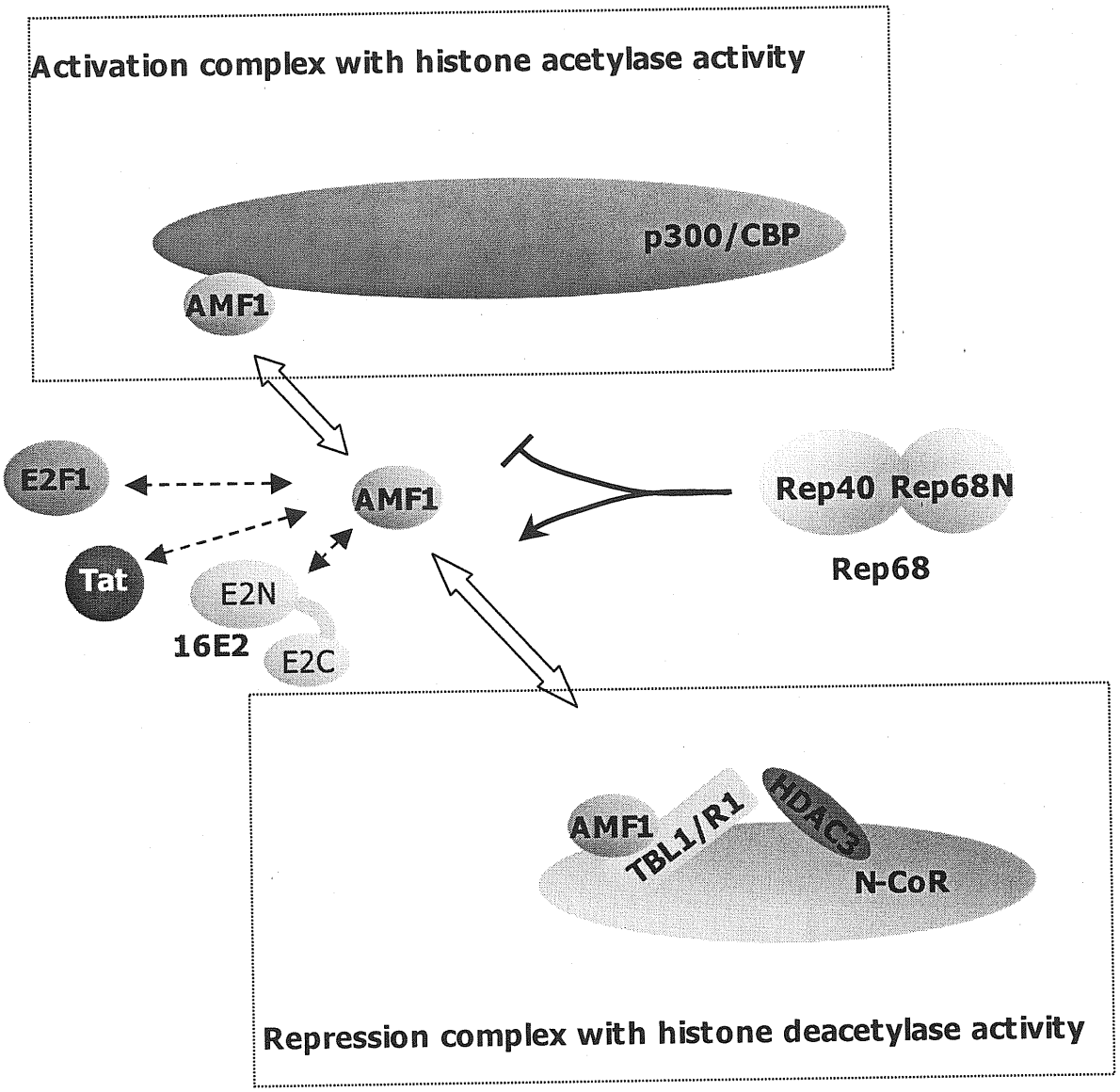


Fig. 4.1) Model for Rep mediated transcriptional repression. AMF-1 can be either in an active complex that includes the HAT p300 or in a repressive complex that is formed by N-CoR, TBL1, TBL1R and HDAC3. Transcriptional activators (hE2F1, HIV-1 Tat and HPV16 E2) utilize Gps2 as a bridging factor to recruit p300 and activate transcription. Rep68, through its C-terminal domain, disrupts the interaction between p300 and AMF-1, thus shifting the equilibrium toward the formation of a repressive complex that is endowed with deacetylase activity.

activity of key regulators of cell cycle such as hE2F1 and of the Tat protein, which is fundamental for reactivation of HIV-1.

4.2 Cellular protein partners of Rep68.

Rep is the key factor that regulates the AAV life cycle and interacts with several proteins that influence the final outcome of AAV infection. A systematic approach for the identification of such proteins has never been tackled. Consequently, the main aim of this second part of the thesis was to develop a tagged proteomic approach for the identification of protein partners for Rep.

The expression of Flag-Rep68 and the purification of its cellular partners in human cells led to the identification of novel interacting proteins (Fig 3.10 and Tab 3.1): DNA-PKcs, KU80, KU70, HSP60 and ANP32B. We could not detect many of the cellular proteins previously described to be interacting with Rep: Rb, RPA and PC4 as an example; and even AMF-1, the protein described in the first part of the thesis. One explanation is that the experimental conditions that we used might be different from those used in other reports. Particularly, the lysis buffer composition could have affected the binding of some of these factors. Another explanation is that the *in vivo* pull-down experiments were conducted in the absence of a helper stimulus. For this reason it will be extremely interesting to repeat this set of experiments in the presence of co-infecting Adenovirus or with pre-treatment of the cells with DNA damaging agents such as UV, camptotheticin or hydroxyurea. Alternatively, while expressing Rep in human cells by transiently transfecting a CMV-driven expression plasmid presents some straightforward practical advantages (The experiment can be easily scaled up to meet the needs of Mass Spectrometry, no virus is produced or manipulated, no need for an helper stimulus), it is now clear that Rep over-expression from a plasmid might not exactly mimic the conditions of a productive AAV infection, and thus might not lead to the comprehensive isolation of all the protein complexes formed by Rep in the cell. For this reason in the future we will design and produce a recombinant AAV (i.e.: a rAAV that carries Flag-Rep under the control of the p5 promoter), which will be used to infect cells.

4.3 Rep and the DNA-PK complex.

Three of the proteins that co-immunoprecipitated with Rep (DNA-PKcs, KU80, KU70) constitute the DNA-PK complex that is involved in the repair of double strand breaks by non-homologous end joining (NHEJ). While it is likely that DNA damage and repair pathways are involved in the processing of AAV genomes, at the moment we cannot envisage a clear role for the interaction between Rep and the DNA-PK complex. We can imagine several possible causes why this physical interaction is DNA-dependent *in vivo*. First, considering that Ku proteins are very abundant in the cell and that can bind DNA ends at high affinity, Rep might co-precipitate along with DNA fragments bound by Ku in the cell. This event might also take place after the sonication step of the immunoprecipitation. Second, the endonuclase activity of Rep might favor the recruitment of the DNA-PK complex near the DNA lesion. Despite the possible artifactual source of Rep/DNA-PK interaction, several works showed that DNA-PK might be involved in the formation of circular concatamers of rAAV genomes *in vivo* (Duan et al., 2003; Song et al., 2001) and in AAV integration (Song et al., 2004). For these reasons, we cannot *a priori* exclude a role for DNA-PK in regulating Rep functions.

4.4 Rep and ANP32 proteins.

ANP32B is the other specific protein identified in Rep68 immuno-complexes from HEK293 cells by Mass Spectrometry (Fig 3.10). We kept investigating the possible role of this interaction in the regulation of AAV biology because it was strong, specific and reproducible *in vivo* and *in vitro* (Fig. 3.13, 3.14, 3.15). Furthermore, we early demonstrated that, in contrast to KU70, Rep binds specifically ANP32B in a DNA-independent manner (Fig. 3.13).

ANP32 (Acidic Nuclear Protein 32) is a family of proteins that plays several roles in the regulation of diverse cellular pathways. In humans there are three different genes (ANP32A, ANP32B and ANP32E) that encode for three different proteins,

which share extensive homology in terms of primary sequence. These proteins are about 250 aa long and can be roughly subdivided into two different domains. At the N-terminus several Leucine Rich Repeats (LRR) are present. This domain is present in a wide variety of cellular proteins and is probably a module for protein-protein interactions (For a recent review see (Kobe and Kajava, 2001)). The last 90 aa at the C-terminus of ANP proteins form an acidic tail. By looking in the sequence databases, the only proteins that share such domain are the chromosomal non- histone protein HMGB1 A and B (High Mobility Group Box 1). The function of this domain is not clear, but, at least in HMGB1, it seems to regulate the interactions of this protein to histones (Ueda et al., 2004).

ANP32 protein expression is developmentally regulated in rat and mouse cerebellum, and has been shown to play a role in the differentiation process of Purkinje cells (Mencinger et al., 1998; Mutai et al., 2000; Opal et al., 2003).

It is still very difficult to reach a consensus conclusion on the main function played in by these proteins in the cellular context: depending on the techniques used, ANP32 proteins have been shown to be specific inhibitors of phosphatase activity (Li et al., 1996), to be part of the INHAT (Inhibitor of Histone Acetyltransferase) multiprotein complex (Seo et al., 2001), to promote apoptosis by enhancing the activity of Caspase-3 and Caspase-9 (Jiang et al., 2003) and to participate in the transport of mature mRNA to the cytoplasm by binding to HuR, a ubiquitously expressed member of the ELAV (embryonic lethal abnormal vision) family of RNA binding proteins (Brennan et al., 2000).

As for the participation of ANP32B in the INHAT complex, Chakravarti and colleagues were able to show that ANP32A and ANP32B formed a quaternary complex with the SET-Iα and SET-Iβ that specifically inhibits the HAT activity of p300 on H3 and H4 histones by binding to their hypoacetylated N-terminal tails (Seo et al., 2002; Seo et al., 2001). Moreover, it seems that ANP proteins directly bind to the N terminal tail of histone H3 and that this binding is modulated by post-translational modifications (such as acetylation, methylation or phosphorylation) (Kutney et al., 2004; Schneider et al., 2004).

It is also worth mentioning that ANP32A was isolated as an E1B55kD/E4ORF6 interacting protein in Adenovirus-5 infected cells and that this interaction strictly depends on the concomitant expression of E4ORF6 (Harada et al., 2002).

One of the most striking observations of our proteomics and co-iimmunoprecipitation data is the preference of Rep68 for binding ANP32B rather than ANP32A. Two possible, non-mutually exclusive explanations can be put forward to rationalize these experimental observations. First, Rep68 might bind the acidic tail of ANP32B, the C-terminal region of the protein that displays a lower similarity to ANP32A in terms of primary sequence (Fig. 3.12a). This hypothesis seems to be corroborated by the fact that a C-terminal deletion mutant of ANP32B lacking the acidic tail (ANP32BΔC, Fig 3.17a) is not capable of binding Rep68 (Fig 3.17c). Alternatively, the different migrating behavior of the two phosphoproteins on the gel might suggest that they differentially undergo post-translational modifications retained in the reticulocyte lysate used for *in vitro* binding assays, and that this difference might influence the affinity for Rep68.

In a further effort to delimitate the protein domains involved in the interaction, we determined that the first 225 aa of Rep68 are responsible for the interaction with ANP32B. This part of the protein is fundamental for replication (Hermonat et al., 1984; Ni et al., 1994) and integration because it binds and cleaves DNA (Im and Muzyczka, 1990). Therefore we believe that the interaction with ANP32B may be important in regulating these processes.

In line with this hypothesis, when tested in AAV replication assays, ANP32 over-expression increased the quantity of replicated rAAV genomes by at least 2.5 fold (Fig. 3.18a and 3.18b). The effect of ANP32 proteins on Rep activities cannot be attributed to the increase of protein production or stability, since the levels of Rep proteins were equal in all the samples of the AAV replication assay (Fig 3.18c). Rather, we think that ANP32 increases the efficiency by which Rep acts as a replication factor by a yet unknown molecular pathway.

We also observed that over-expression of ANP32 proteins increases the viral titer of AAV vectors produced under standard conditions (Fig 3.21). This last evidence not only confirms the results of AAV replication assays, but also establishes that, in these experimental conditions, the increase in replication can be converted in an

increase in packaged genomes. From the biotechnological standpoint, an increase of 2.5-3 fold in yield means that the same amount of vector can be produced by using one third of cell culture material (Cells, transfection reagents, plasmids, culture dishes etc. etc), thus achieving a cost cut of at least 60%. We then believe that co-expression of ANP32 together with the normal set of plasmids for the production of AAV vectors could have an impact in improving the efficiency of their production.

The fact that both ANP32 proteins have an effect on AAV replication and vector production, even though only ANP32B binds directly Rep68 (Fig 3.13, Fig 3.14, Fig 3.15), might be an indication that they impinge on a common cellular pathway important for this activity. In fact, in support of this hypothesis, several reports in the literature show that ANP32A and ANP32B can be purified together as a protein complex (Brennan et al., 2000; Jiang et al., 2003; Seo et al., 2001). On the contrary, by knocking down ANP32B expression AAV replication is partially inhibited (Fig. 3.20), demonstrating that this cellular factor is specifically involved in this process. Interestingly, in line with the biochemical data (Fig 3.13, Fig 3.14, Fig 3.15), AAV replication is inhibited only in the absence of endogenous ANP32B, while silencing of ANP32A has no effect.

More recently an inhibitory effect of Ad-5 VAI RNA on the export and processing of hairpinned siRNA was reported (Lu and Cullen, 2004). Therefore, it will be extremely important to assess ANP32A and ANP32B endogenous protein levels in the experiment shown in Fig. 3.20. This will rule out the possibility that the VAI RNA expressed by pDG impair or reduce the silencing effect of the pSuper co-transfected plamids.

Our AAV replication experiments were conducted in the presence of Cap proteins provided *in trans* by the pDG plasmid. To rule out the possibility that ANP has an effect on the process of AAV genome incapsidation rather than replication, we will perform these experiments in the absence of capsid proteins. Furthermore the AAV replication experiments were done in the presence of Adenovirus helper genes provided by pDG, while the co-immunoprecipitations were not. Accordingly, it would be interesting to assess whether the helper functions provided by Adenovirus genes change the stoichiometry of the Rep/ANP protein complex (i.e.

whether Rep can from a ternary complex with ANP32A and ANP32B). For this reason we plan to perform co-immunoprecipitation experiments between Rep and ANP proteins in the presence of Ad E4ORF6 in HEK293 cells (Which already produce Ad E1B55kD (Harada et al., 2002)).

To summarize, the results obtained by either over-expressing ANP or silencing their expression (Fig. 3.18 and Fig. 3.20) can be interpreted in this way: ANP32A and ANP32B work in the same pathway and they can substitute for one another. In support of this hypothesis, ANP32A$^{-/-}$ mice are viable and do not show any phenotypic abnormalities (Opal et al., 2004), an observation that suggests a redundancy in terms of functions among ANP32 family members. Furthermore, both ANP32A and ANP32B exert a positive effect, possibly together as a protein complex, in the same molecular pathway that regulates AAV replication. In particular, we propose that over-expressed ANP32A, even though it does not bind Rep directly, has an effect on AAV replication (Fig. 3.18) because it can associate endogenous ANP32B. At the moment we cannot automatically exclude the presence of endogenous ANP32A in the Rep containing complexes, maybe by indirect interaction through ANP32B. In contrast, only silencing of endogenous ANP32B causes a decrease in AAV replication because in this situation, even if present, endogenous ANP32A cannot be bound by Rep68.

4.5 Possible functional roles of ANP32 proteins in regulation of Rep function.

We observed that Rep68 specifically interacts with ANP32B both *in vitro* and *in vivo* and that ANP32 proteins are involved in AAV replication. What might be the role of these proteins in AAV biology and why does Rep bind them?

Several molecular mechanisms might underlie our experimental observations in AAV replication. Nonetheless, from the recent scientific literature presented above a main indication is emerging among the plethora of cellular functions that have been ascribed to these proteins: ANP32 are linked to chromatin structure regulation. This might affect AAV replication, since it is known that upon

Adenovirus infection the AAV genome is indeed partially chromatinized (Marcus-Sekura and Carter, 1983).

First, in the Rep context the ANP32 family might have similar functions to HMG proteins. In support of this hypothesis we can recall that ANP32 proteins and HMGB1 share functional domains, are both chromatin associated and both interact with Rep (Fig. 3.13, 3.14, 3.15) and (Costello et al., 1997; Kutney et al., 2004; Schneider et al., 2004). Similarly to HMGB1 and RPA (Stracker et al., 2004), ANP32 might increase the binding of Rep to DNA in the chromatin context, thus augmenting its efficiency in AAV replication. This model would explain the positive effect on AAV replication observed by overexpressing ANP32 proteins.

Second, the picture might be even more complex considering that in HeLa nuclear extracts ANP32 proteins can form a protein complex together with SET-Iα and SET-Iβ (Seo et al., 2001), proteins that are part of the Nucleosome Assembly Proteins family, involved in the active assembly of histones onto DNA. This might mean that Rep directly bind ANP32B, but also recruits a complex that is capable of assembling/disassembling histones from chromatin. In the context of AAV this nucleosome assembly activity might facilitate Rep initiated replication on chromatinized templates. Similarly, Set-I, also known as TAF-I (Template activating factor I), was originally discovered because it enhances Adenovirus replication from a chromatinized template (Matsumoto et al., 1995; Nagata et al., 1995a).

Finally, what can be the role of ANP32A in the Ad-5 E1B55kD/E4ORF6 protein complex? Is this a possible link between Adenovirus helper functions and the activity of Rep? It is tempting to speculate that the association of Rep with ANP32 proteins (maybe the formation of an active complex that includes ANP32B, ANP32a, SET-Iα and SET-Iβ) is influenced directly or indirectly by the E1B55kD/E4ORF6 protein complex, which has already been described to be sufficient for AAV replication *in vivo* (Stracker et al., 2004). Further experiments will be carried on to demonstrate this hypothesis.

In conclusion, we were able to isolate ANP32B as a novel cellular protein partner for AAV Rep. We also demonstrated that ANP32B is involved in AAV replication and that it can be used as a biotechnological tool to improve rAAV vetors production.

Given the importance of these observations we believe that further study of ANP32B will give new, important insight into the molecular mechanisms that regulate Rep functions and AAV replication.

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Acknowledgements

I would like to thank Mauro Giacca for trusting me and for giving me the opportunity of working in an international, friendly and enthusiastic environment. Thank you also for teaching me to look at scientific problems from a wider perspective.

Thanks to Alessandro Marcello who, besides giving constant scientific tutoring and advices, has constantly supported me during the hardest days, as well as sharing the best moments of excitement for new results and ideas.

I also want to thank Mike Myers for hosting and helping me in every possible way for those long six months overseas. Thank you Mike and Antonella for providing (apart from useful tips and hints that saved me from getting lost on Long Island) the blankets that helped me to overcome those cold nights in Olney...

Thanks to all the people in the Molecular Medicine Lab for jokes, laughs and discussions on topics that were not only scientific. In particular, I want to mention Ramiro, Marina and Laura for always being there when I needed help. Super thanks to Alejandro for solving all the computer problems and for suggesting so many super projects...

Thanks a lot to Barbara Boziglav and Marina Dapas for providing excellent technical assistance.