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The adeno-associated virus Rep78 major regulatory protein forms multimeric complexes and the domain for this activity is contained within the carboxy-half of the molecule

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Abstract The adeno-associated virus (AAV) encoded Rep78 is a multifunctional protein which is able to regulate transcription, is required for AAV DNA replication, and appears necessary for site specific integration of AAV DNA into human chromosome 19. Being analogous to the large T antigen, the replication protein of polyomaviruses which is known to homo-multimerize, it seemed likely that the Rep78 protein would also interact with itself to carry out at least some of its functions. Furthermore, in electrophoretic mobility shift assay studies by many laboratories on Rep78/68 protein interaction with AAV terminal repeat DNA it has been noticed that multiple high bands often result. These data suggest Rep78-Rep78 interaction. In this study it is directly demonstrated that Rep78 *is* able to form multimeric complexes as measured by West-Western and chemical cross-linking assays. Furthermore, using an amino-truncated Rep78 protein, it is demonstrated that the Rep78 homo-multimerization domain is contained within the carboxy-half of the protein.

Key words: Adeno-associated virus; Protein-protein interaction; Rep78

1. Introduction

Adeno-associated virus (AAV) is a helper dependent human parvovirus, which requires that the cell be co-infected with an adeno- or herpes virus to allow for AAV productive infection [1,2]. The Rep78 nuclear protein, encoded by AAV, plays important roles in AAV DNA replication [3,4], AAV chromosomal DNA integration into human chromosome 19 [5,6], the *trans*-regulation of AAV gene expression [7–9], and the *trans*-regulation of various heterologous genes [10–15]. Consistent with Rep78's many biological roles it has many defined biochemical activities. Rep78 is able to recognize secondary structure within DNA (AAV's terminal repeats, TR), bind DNA of a specific sequence ($GCTC^3$), has site specific and strand specific endonuclease activity (nicks AAV DNA at the terminal resolution site [*trs*] within the TR, binds ATP, and possesses an ATP dependent DNA helicase activity [16–20].

The basis for this study was the likelihood that homo-multimerization of Rep78 was needed for at least some of Rep78's numerous functions. It is known that the large T antigen, the analogous nuclear replication protein of polyomaviruses, has many of the same functions as Rep78 [21]. Large T antigen is known to form a double hexamer while binding to the origin of SV40 DNA replication [22]. This resulting complex then has helicase activity which is needed for the initiation of SV40 DNA replication. Large T antigen also binds p53 as a dimer [23].

The finding that in electrophoretic mobility shift assays of Rep78, and the closely related Rep68 protein, interaction with AAV's TRs results in multiple bands has initiated the hypothesis of Rep-Rep protein interaction to form multimers [24]. However, these results are only suggestive and do not directly demonstrate that direct Rep78-Rep78 interactions are taking place. Here, Rep78-Rep78 recognition is directly demonstrated using two different assay systems, West-Western and chemical cross-linking analysis. Furthermore, the Rep78-Rep78 homo-multimerization interaction domain is mapped to be enclosed within the carboxy-half of the molecule.

2. Materials and methods

2.1. Production and purification of ^{35}S-labeled MBP and MBP-Rep78

Rep78 was generated as a fusion protein with maltose binding protein (MBP) using the New England BioLabs protein fusion and purification kit according to the manufacturer's instructions. A detailed protocol for the construction of the pMal-Rep78 and purification of MBP-Rep78 protein is given elsewhere [20]. For the preparation of ^{35}S-labeled MBP-Rep78 chimeric protein essentially the same protocol is used with minor modifications. Briefly, overnight cultures were inoculated again in 10 ml of LB medium, and grown until the absorbance reached A_{600} 0.5. The cells were pelleted and resuspended in minimal medium previously warmed to 37°C. After approximately 10 min growth in minimal medium, 100 μCi of [^{35}S]methionine, 50 μl of 2 mM methionine free amino acid mix (Promega), and IPTG were added to a final concentration of 0.3 mM. Incubation was continued for 30 min with agitation at 37°C, then unlabeled methionine was added to a final concentration of 1 mM and 2 ml of Luria broth. Cells were pelleted, resuspended in column buffer (25 mM Tris, pH 7.5, 200 mM NaCl, 1 mM DTT, and 1 mM EDTA) and stored at −70°C for 30 min. Purification of induced chimeric protein in labeled medium was essentially carried out as mentioned elsewhere [25].

2.2. Cloning and purification of His-Rep78

Rep78 open reading frame from pALTER-AAV-3 [20], on a 1.9 kb *Sph*I and *Xho*I fragment, was cloned in-frame with the 6 histidine tag sequence into an altered pQE-32 expression vector (Qiagen Inc.). The polylinker of the original vector was first replaced with a custom polylinker containing *Sph*I and *Xho*I sites. The resulting construct, pQE-Rep78, produced His-Rep78 protein upon IPTG induction and was purified to 90% homogeneity according to the manufacturer's instructions.

2.3. Construction of amino-truncated Rep78 mutants

Two Rep78 mutants with N-terminal deletions were generated by digesting the pMal-Rep vector [20] with *Xho*I and either *Eco*RI or *Bam*HI. The full length Rep open reading frame is at nucleotides (nt) 321–2185. The *Bam*HI site is at nt 1045 and the *Eco*RI site is at nt 1985. The *Xho*I site is downstream of the Rep ORF at nt 2233. After digesting the plasmid pMal-Rep with either *Bam*HI+*Xho*I or *Eco*RI+*Xho*I, N-terminal deleted fragments were isolated and these were

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again cloned into the pMalc2 expression vector (New England BioLabs), in frame with MBP. These vectors, upon IPTG induction, produced recombinant mutant MBP-chimeric proteins.

2.4. West-Western analysis

Electrophoretically separated proteins were electroblotted onto nitrocellulose as described by Towbin et al. [25]. Additional protein binding sites on the nitrocellulose were blocked by incubating for 30 min at room temperature in 1% protease free BSA prepared in binding buffer (25 mM Tris pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA, 5 mM KCl). All the additional steps of washing and incubation were performed in 0.1% protease free BSA prepared in binding buffer. For West-Western blot analysis, the blots were incubated for 30 min at room temperature with constant rocking in 1:10 000 anti-MBP (New England BioLabs) antibodies, or 1:200 dilution of anti-Rep antibodies (a generous gift from Dr. James Trempe) raised in rabbit. Again the blots were subjected to three washes of 10 min each with constant stirring. Incubation with 1:10 000 dilution of anti-rabbit antibodies conjugated to horseradish peroxidase (HRPO) was carried out for 30 min at room temperature with rocking. After extensive washing, blots were incubated in peroxidase enzymatic assay solution consisting of 5 mg of 4-chloronaphthol substrate and 10 μl of H_2O_2. After the appearance of the bands the blot was thoroughly rinsed with water and dried. For chemiluminescence assays, after the incubation of the blots with HRPO conjugated anti-rabbit antibodies, the blots were extensively washed and incubated in Luminol substrate solution with enhancer (Renaisense, DuPont) for 1 min. Dried filters were exposed to films for varying lengths of time to get appropriate signal. For West-Western analysis, after the BSA blocking step, filters were incubated either in ^{35}S-labeled MBP or in ^{35}S-labeled MBP-Rep78 for 30 min prepared in 1% protease free BSA in binding buffer with constant rocking. Then the filters were given a single wash in binding buffer, dried and autoradiographed. West-Western analyses were also performed with either unlabeled MBP (10 μg/ml) or unlabeled MBP-Rep78 (10 μg/ml). It is similar to the Western analysis except for the incubation of the cold protein before probing with antibodies. Rep78 binding proteins on the blots were visualized by HRPO enzymatic assays as was done in the case of Western blot analysis.

2.5. Protein-protein cross-linking with dithiobis sulfosuccinimidylpropionate (DTSSP)

In a final volume of 50 μl (PBS, 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.5), 5 μg of Rep78 was incubated with different concentrations of DTSSP ranging from 0.2 to 6.4 mM for 30 min at room temperature. The reaction was terminated by the addition of an equal volume of 1 M Tris pH 7.5 and the products were separated on 8% SDS-PAGE. Gels were stained with Coomassie brilliant blue.

2.6. Generation of ^{35}S-labeled nuclear extracts

Generation of ^{35}S-labeled nuclear cellular extracts from HeLa cells was carried out as described by Owens et al. [26], with the following alterations. Briefly, HeLa cells were grown to 80% confluence in 100 mm plates in the presence of 7% fetal calf serum and were infected with adenovirus type 2 (multiplicity of infection (MOI) of 5) and AAV (MOI of 5) when appropriate. Medium was aspirated, the cells washed twice with methionine free medium and incubated in the same for 4 h in a CO_2 incubator in the presence of 200 μCi of [^{35}S]methionine. Cells were washed thrice in PBS and incubated in the same buffer for 30 min in the presence of 1 mM EDTA. Cells were scraped off in 2 ml of PBS, centrifuged, and resuspended in 1 ml of buffer containing 0.25 M sucrose, 10 mM Tris-HCl, 10 mM $MgCl_2$, 0.5% Nonidet P40, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 1.0 mM DTT. After 10 min of incubation the cells were sheared with six strokes of a Dounce homogenizer using pestle B. The nuclei were then pelleted by centrifugation for 5 min at 1800×*g* and resuspended in 300 μl of 50 mM HEPES-KOH (pH 7.5), 1 mM PMSF, and 0.5 mM DTT, frozen at −70°C, thawed on ice and adjusted to 600 mM NaCl. After 1 h on ice the debris were pelleted by centrifugation at 20 000×*g* for 1 h. Finally, glycerol was added to 20% and the nuclear extracts frozen at −70°C.

2.7. Rep78 affinity chromatography

Selection of Rep78 binding cellular proteins from the HeLa cell ^{35}S-labeled nuclear extracts was carried out by Rep78 affinity chromatography using MBP-Rep78 fusion protein bound to amylose resin. Amylose resin, 0.5 ml (New England BioLabs) extensively washed in binding buffer (0.25 mM Tris pH 7.8, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl), was incubated with 5 μg of MBP-Rep78 fusion protein at room temperature for 30 min with constant stirring, and loaded onto a column. The Rep78-resin was extensively washed and then 10 μg of ^{35}S-labeled HeLa cell nuclear extract was incubated with the MBP-Rep78/amylose resin in 1 ml binding buffer. After extensive washing, bound proteins were eluted along with MBP-Rep78 with 10 mM maltose in binding buffer. These proteins were subjected to 10% SDS-PAGE, and the gels were dried and autoradiographed.

3. Results

3.1. Rep78 binds to itself as determined by West-Western analysis

Many transcriptional factors have the ability to form dimers and higher order complexes. In some cases multimeric complexes are required for biological activity, such as the E2 transactivator protein of the papillomaviruses [27]. The possibility of Rep78 interacting with itself was tested by two direct methods. First, Rep78-Rep78 interaction was explored using West-Western analysis. MBP-Rep78 was electrophoretically separated, blotted onto nitrocellulose membranes and probed with purified [^{35}S]MBP-Rep78 probe. As shown in Fig. 1A there was a dosage dependent increase in the binding to Rep78. This interaction was not observed when the membrane was probed with labeled MBP (data not shown). Next, in Fig. 1B, similar blots were also subjected to a chemiluminescence assay to document dosage dependent Rep-Rep interactions. However, in this case the target protein was SDS-PAGE/blotted His-Rep78. His-Rep78 was produced by cloning the Rep78 ORF into the pQE-32 vector and purified from bacteria as described in Section 2. The blots were then incubated

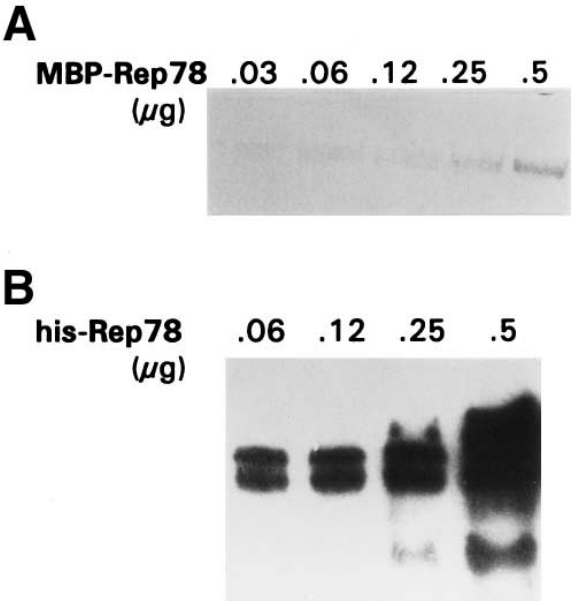

Fig. 1. Rep78 binds to itself as determined by West-Western analysis. A: Increasing concentrations of MBP-Rep78 was applied onto 8% SDS-PAGE and the separated protein was blotted onto nitrocellulose paper. After probing with labeled MBP-Rep78, blots were autoradiographed. B: Increasing concentrations of His-Rep78 were electrophoretically separated on 8% SDS-PAGE and blotted onto nitrocellulose paper. Chemiluminescence assay was conducted with MBP antibodies after incubation with MBP-Rep78.

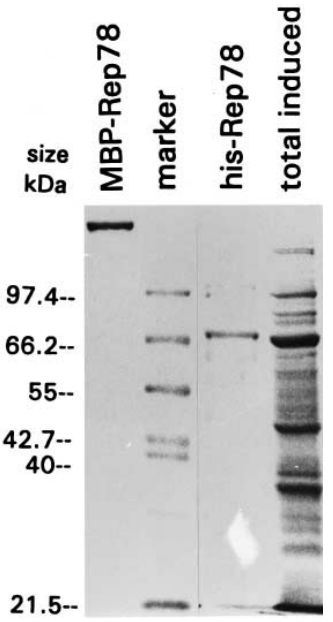

Fig. 2. Production of purified MBP-Rep78 and His-Rep78 chimeric proteins. The Rep78 open reading frame was cloned into the pMalc2 vector to produce MBP-Rep78 protein upon induction (New England Biolabs Protein Purification System) and purified per kit instructions. The Rep78 open reading frame was also cloned into pQE-31 to produce his-Rep78 upon induction (Qiagen) and purified per kit instructions. The purified chimeric proteins were analyzed on 8% SDS-PAGE, gels were dried and Commassie blue stained. The lanes were loaded as indicated. The lane indicated as 'total induced' refers to total protein from the his-Rep78 induced *E. coli*.

with MBP-Rep78 and probed with anti-MBP antibodies. Examples of bacterially produced and purified MBP-Rep78 and His-Rep78 are shown in Fig. 2. Without Rep78-Rep78 interaction, chimeric MBP-Rep78 probe could not bind the His-Rep78 target. These data indicate direct interaction, homo-multimerization, between Rep78 proteins.

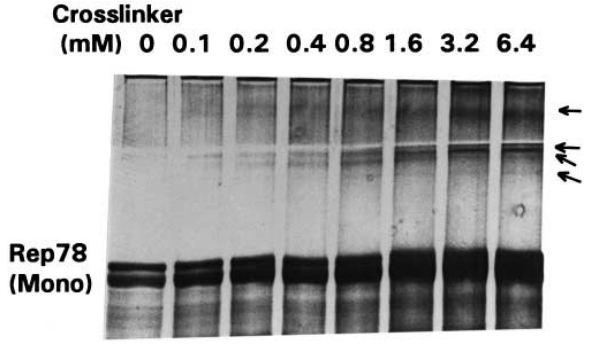

Fig. 3. Rep78 forms multimeric complexes as determined by chemical cross-linking. Chemical cross-linking of MBP-Rep78 with DTSSP. Rep-78 (5 mg/ml) was incubated in 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.5 (PBS) along with different concentrations of DTSSP as indicated. Reaction was terminated with an equal volume of 1 M Tris, pH 7.5 and applied onto 8% SDS-PAGE. Arrows indicate the formation of higher order complexes (dimer, trimer, etc.) of MBP-Rep78. As a favored degradation product of MBP-Rep78 is also present in the reaction, some of the crosslink-generated products bands may include these species, thus complicating the interpretation of these data.

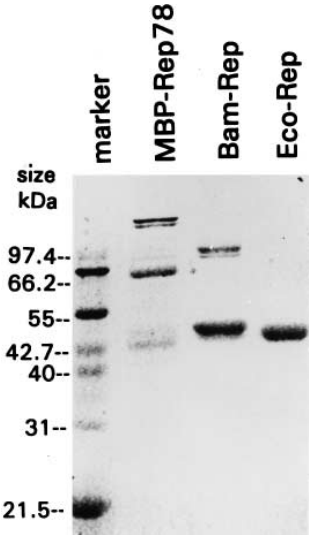

Fig. 4. Production and purification of wild type and N-terminal deleted mutant recombinant MBP-Rep chimeric proteins. Cloned mutant proteins were expressed in *E. coli* and purified by affinity chromatography on amylose resin as mentioned in Section 2. Purified proteins were subjected to 10% SDS-electrophoresis and stained with Coomassie brilliant blue. The marker lane includes 6 prominent proteins with molecular weights of 97.4, 66.2, 55, 42.7, 40, 21.5 and 14 kDa. Low molecular weight bands in both Bam-Rep and wild type Rep (MBP-Rep78) are favored degradation products of the full length recombinant proteins.

3.2. *Rep78 binds itself to form multimeric complexes as determined by chemical cross-linking analysis*

Rep78-Rep78 interaction was also investigated by chemical cross-linking. Cross-linking experiments were performed with DTSSP which is a homo-bifunctional reversible cross-linking reagent. As shown in Fig. 3, [^{35}S]MBP-Rep78 was cross-linked with itself by this reagent as shown by the appearance of new high bands. With the increasing concentrations of DTSSP, increasingly intense higher order complexes were observed. This covalent cross-linking is possible when the molecules interact with each other in solution to allow the homo-bifunctional DTSSP agent to carry out covalent cross-linking. These data further indicate that Rep78 interacts with itself and forms higher order complexes. Due to the large sizes of these complexes accurate measurements of their apparent molecular weights are not possible, and thus the number of MBP-Rep78 molecules in these complexes cannot be accurately determined. Furthermore, breakdown products of full length MBP-Rep78 may still be able to homo-multimerize, further complicating the analysis.

3.3. *The Rep78-Rep78 interaction domain maps to the carboxy-half of the molecule*

To map the homo-multimerization domain(s) of Rep78 two amino-terminal deleted versions of Rep78 were constructed. Bam-Rep (deleted to the *Bam*HI site at nt 1045) deletes the 241 amino-terminal amino acids, while Eco-Rep (deleted to the *Eco*RI site at nt 1985) deletes to amino acid 554. These truncated Rep78 versions were cloned into the same expression vector which produced MBP-Rep78 fusion protein. The truncated proteins were induced, ^{35}S-labeled, and purified, as shown in Fig. 4. These truncated Rep probes were then compared to full length MBP-Rep78 in West-Western assays for binding to membrane bound MBP-Rep78. As shown in Fig. 5,

three equivalent membranes were each probed with a different probe. Note that the Bam-Rep probe was able to recognize and bind MBP-Rep78, while the Eco-Rep probe was not. These data strongly suggest that the carboxy-half of Rep78 contained the homo-multimerization domain, and that the terminal 66 amino acids were insufficient for Rep78-Rep78 binding.

3.4. *The Rep78 protein binds to specific nuclear proteins present in adenovirus and AAV infected cells by Rep78 affinity chromatography*

Rep78 is a nuclear protein, thus we next observed if Rep78 was able to recognize nuclear proteins specifically present in AAV plus adenovirus infected cells. The binding of Rep78 to adenovirus and AAV specific cellular proteins was assayed by Rep78 affinity chromatography. A chromatography column with bound Rep78 was first generated by incubating MBP-Rep78 protein in an amylose resin column as described in Section 2. In vivo ^{35}S-labeled HeLa cellular extracts were passed through the column, the column washed, and then bound protein eluted with 10 mM maltose and 100 mM NaCl and analyzed by 10% SDS-PAGE. The results are shown in Fig. 6. As seen in this figure there are multiple new proteins which are selected by binding Rep78 in the Rep78 selected HeLa+Ad+AAV lane as compared to the Rep78 selected HeLa+Ad lane. One new AAV specific protein, labeled #1 in Fig. 6, is of a size consistent with Rep78 (78 kDa). Another Rep78 selected protein, labeled #2 in Fig. 6, is of a size consistent with the major capsid protein VP3 (61 kDa). The faint band(s) present above the putative Rep78 band (#1) may be the minor capsid VP1 (87 kDa).

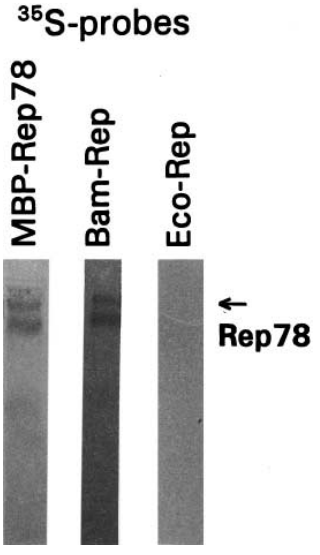

Fig. 5. The Rep78-Rep78 interaction domain is fully included within the carboxy-half of Rep78. West-Western analysis of MBP-Rep78 electrophoretically separated, electroblotted, and probed with one of three ^{35}S-labeled Rep probes (wild type MBP-Rep78, Bam-Rep, and Eco-Rep mutant protein probes). Note that both the wild type MBP-Rep78 and Bam-Rep probes were able to recognize membrane bound MBP-Rep78, while Eco-Rep was not. The low mobility band below MBP-Rep78 is a degradation product of the full length protein.

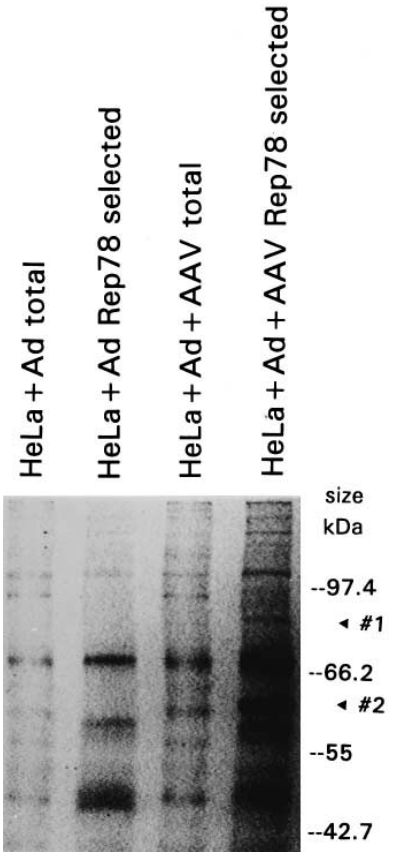

Fig. 6. Rep78 binds multiple AAV specific nuclear proteins in adenovirus and AAV infected cells. HeLa cells were labeled with [^{35}S]methionine and nuclear extracts made. These nuclear extract were then selected by Rep78 affinity using amylose resin. The selected proteins were then analyzed by 8% SDS-PAGE, along with samples of total nuclear protein, and then autoradiographed. Only the top half of the autoradiograph is shown to allow greater detail. No obvious differences were observed between the two selected protein populations in the bottom half of the gel. Notice that multiple new proteins are selected in the HeLa+Ad+AAV lanes as compared to the HeLa+Ad lane. The new AAV specific protein labeled #1 is of a size consistent with Rep78 (78 kDa), while the protein labeled #2 is of a size consistent with the major capsid protein VP3 (61 kDa). The faint band(s) present above the putative Rep78 band (#1) may be the minor capsid VP1 (87 kDa).

4. Discussion

This study directly demonstrates that AAV Rep78 forms homo-multimers by both West-Western and chemical cross-linking analysis. We believe that this self recognition is to be expected due to Rep78's multiple roles in transcriptional regulation [7–15] and DNA replication [3,4]. Rep78's interaction with DNA has been studied extensively [6,16,17,28–32], but its potential protein-protein interactions have not. Thus far the Rep78 protein has been shown to bind the AAV capsid proteins [33] and to the cellular transcription factor Sp1 [13]. We had several reasons to suspect that Rep78 is able to form homo-multimers. First, it is known that some cellular and viral transcription factors form homo-multimers, such as papillomavirus E2, which then have a biological activity [27,34]. Second, Rep78 is highly analogous to large T antigen, the equivalent replication protein of the polyomaviruses. Large T antigen has many of the same functions as Rep78 and is known to form multimeric complexes [21–23]. Finally, obser-

vations that electrophoretic mobility shift assays (EMSA) of Rep68 and Rep78 protein interaction with AAV's TR DNA results in multiple bands suggested Rep-Rep protein interaction [26]. However, these latter results are only suggestive of Rep-Rep protein interaction and do not directly demonstrate that such interactions are taking place. Such multiple banding in the EMSA assays could be caused by induction of alternative DNA secondary structure due to Rep78 binding, or Rep78 binding to multiple, different sites. The TRs are a complex series of palindromes and many variant structures can form. In this study Rep78-Rep78 recognition is directly demonstrated using two different assay systems, West-Western and chemical cross-linking analysis. These data indicate that Rep78-Rep78 recognition is real. Although we cannot presently estimate the maximum number of Rep78 molecules which are able to stably complex together, the size and number of high bands present at the highest level of DTSSP in Fig. 3 suggest that complexes of dimer and larger size are being formed.

It has generally been found that the protein binding domains are often clearly demarcated, located at either the N-terminal or the C-terminal end for many transcriptional factors [27,34,35]. The ability of Bam-Rep to bind MBP-Rep78 indicates that the homo-multimerization domain is fully contained within the carboxy-half of Rep78. In another related study we demonstrate that Rep78 is also able to bind to a number of cellular proteins, and that most of these interactions require the amino-half of Rep78 (Hermonat and Batchu, submitted). Thus, these data suggest that Rep78 may be able to simultaneously bind cellular proteins and itself. Although we do not have the evidence of specific biological activities for Rep78 multimers, it is likely that this activity is a prerequisite for Rep78's interaction with certain substrates. The identification of Rep78 mutants which do not homo-multimerize or bind wild type will allow us to determine the significance of this activity. Possibly Rep78 multimerization is required for Rep78-cellular protein or Rep78-DNA interaction.

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