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Accurate single-day titration of adenovirus vectors based on equivalence of protein VII nuclear dots and infectious particles

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Summary

Protein VII is an abundant component of adenovirus particles and is tightly associated with the viral DNA. It enters the nucleus along with the infecting viral genome and remains bound throughout early phase. Protein VII can be visualized by immunofluorescent staining as discrete dots in the infected cell nucleus. Comparison between protein VII staining and expression of the 72 kDa DNA binding protein revealed a one-to-one correspondence between protein VII dots and infectious viral genomes. A similar relationship was observed for a helper-dependent adenovirus vector expressing green fluorescent protein. This relationship allowed accurate titration of adenovirus preparations, including wild-type and helper-dependent vectors, using a one-day immunofluorescence method. The method can be applied to any adenovirus vector and gives results equivalent to the standard plaque assay.

Keywords

Adenovirus; viral titer; protein VII; gene therapy; adenovirus vectors

1. Introduction

Several methods exist for accurate titration of adenovirus stocks. These include the plaque assay and other assays that depend on viral replication, such as the fluorescent cell-counting procedure (Philipson, 1961; Tollefson et al., 2007). With such methods first generation E1-deleted expression vectors can be assayed only in E1-expressing cell lines. The development of non-replicating helper-dependent (HD) vectors has necessitated the establishment of assays that do not rely on replication. DNA detection methods including Southern blot, slot blot and real-time PCR have been developed for this purpose (Crettaz et al., 2008; Kreppel et al., 2002; Ma et al., 2001; Palmer and Ng, 2004; Puntel et al., 2006).

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A convenient and rapid alternative method for titration of any adenovirus stock, including E1-deleted and HD vectors takes advantage of the presence of the viral protein VII, which is a necessary component of all viral preparations and is bound tightly to viral genomic DNA during the early stages of infection. The adenovirus nucleoprotein core consists of double stranded genomic DNA, three highly basic viral proteins VII, V, and μ (mu), as well as protein IVa2 and the 55-kDa terminal protein (Amin et al., 1977; Brown et al., 1975; Hosokawa and Sung, 1976; Maizel et al., 1968; Prage and Pettersson, 1971; Rekosh et al., 1977; Russell et al., 1968; Weber et al., 1983; Zhang et al., 2001). Protein VII is the major protein component of the core, with an estimated 1,070 copies present per virion (Everitt *et al.*, 1973). Within the virion protein VII and the viral genome form a highly compact complex that also includes protein V and μ .

The nature of the transition from tightly compacted core to transcriptionally active viral chromatin is not understood. Prior to the action of the viral E1A transcriptional activator, the great majority of the viral genome is transcriptionally silent (Flint and Shenk, 1997). Protein VII can mediate significant DNA condensation and transcriptional repression in vivo, suggesting that this protein is important in the configuration and function of viral chromatin as it is released into the nucleus (Johnson *et al.*, 2004).

When infected cells are analyzed by immunofluorescent staining, protein VII is observed as discrete dots localized exclusively in the host cell nucleus, appearing within the first 60 minutes of infection (Xue *et al.*, 2005). The dots remain throughout early phase and their intensity decreases after this with the progress of infection (Chen et al., 2007; Xue et al., 2005).

Several lines of evidence indicate that during early phase, transcriptionally active viral chromatin contains viral DNA in complex with protein VII. First, protein VII is found in the nucleus throughout early phase and chromatin immunoprecipitation experiments demonstrated that viral DNA is associated with protein VII during this time (Chen et al., 2007; Xue et al., 2005). Second, protein VII can associate with the viral transcriptional activator E1A, which controls early viral gene activation (Johnson *et al.*, 2004). Third, protein VII associates with the cellular histone chaperone SET/TAF- β , which is required for maximal early viral transcriptional activation (Haruki et al., 2003; Haruki et al., 2006; Xue et al., 2005). Finally, protein VII is localized in the infected cell nucleus in discrete dot structures whose appearance coincides with early phase transcription (Chen et al., 2007; Xue et al., 2005).

During previous studies it was observed that the number of protein VII dots in host cell nuclei appeared to closely correspond to the multiplicity of infection, suggesting that each dot represents a single infectious viral genome. This report documents that there is in fact a one-to-one relationship between protein VII dots and incoming infectious viral particles. These data support a model in which protein VII plays an important and ongoing role in the function of transcriptionally active viral chromatin. Importantly, the correspondence between protein VII dots and infectious genomes allows quantification of nuclear dots as a means for determining the concentration of infectious viral particles in a preparation. Since protein VII is a necessary component of all adenovirus vector preparations, this method has utility in the quantification of infectivity of helper-dependent stocks, for which standard plaque assays can not be undertaken, as well as rapid titration of other adenovirus vectors.

2. Materials and Methods

2.1 Cells and viruses

HeLa cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (NCS), penicillin, and streptomycin (all from GIBCO). Where indicated, cells were treated with hydroxyurea (HU, 10 mM), phorbol 12-

myristate 13-acetate (PMA, 50 ng/ml), and forskolin (0.2 mM). 293 cells were grown as monolayers in Minimum Essential Medium Alpha 1X (MEM Alpha) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Adenovirus type 5 dl309 (Jones and Shenk, 1979) was propagated in HeLa cells to produce virus stocks. The stock particle concentration for all experiments was 4.6×10^{12} particles per ml. Physical measurement of particles was obtained using optical absorbance (OD₂₆₀) of disrupted virions according to a published procedure (Mittereder et al., 1996). Adenovirus type 5 Reference Material was obtained from the ATCC (catalogue #1516). Helper-dependent virus was generated using a CreloxP system obtained from Microbix (Chen *et al.*, 1996; Parks *et al.*, 1996; Sandig *et al.*, 2000). This consists of plasmid pC4HSU, into which expression cassettes are cloned, Cre recombinase-expressing 293Cre4 cells, and helper virus H14, which is an E1-deleted adenovirus containing loxP sites flanking the packaging signal (Sandig *et al.*, 2000). Plasmid pC4HSU/GFP was digested with Pme I, transfected into 293Cre4 cells, and helper H14 was added to generate vector gAd.HSU/GFP. Details of the rescue and amplification process have been described previously (Witting *et al.*, 2008). The virus was purified by CsCl step gradient centrifugation followed by CsCl isopycnic separation. The helper-dependent adenovirus band was collected and dialyzed against 10 mM Tris-HCl (pH7.5), 1 mM MgCl₂, 150 mM NaCl and 10% glycerol. The level of contamination with helper was determined by plaque assay and shown to be lower than 0.03% (Witting *et al.*, 2008).

2.2 Immunofluorescence

HeLa cells were grown on 22- by 22-mm glass coverslips (Fisher) for 24 hours prior to infection. Cells were incubated with virus for 1 hour as described previously (Johnson *et al.*, 2004). Cells were then washed two times in PBS to remove unadsorbed virus, followed by incubation for an additional 3 hours in DMEM plus 10% NCS at 37°C. After incubation the cells were washed with PBS supplemented with 1.5 mM MgCl₂ (PBS+) and fixed with pre-chilled 100% methanol for 10 minutes at -20°C, followed by air drying. Cell staining was performed essentially as described previously (Ornelles and Shenk, 1991). The source of the protein VII polyclonal antibody was as described (Johnson *et al.*, 2004). Cells were rehydrated in PBS+ three times for 5 minutes followed by incubation in blocking buffer (25 mM Tris [pH 8.0], 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl₂, 2.5% bovine serum albumin, 13 mM glycine, 0.05% Tween 20 and 20% goat serum) for 1 hour. Cells were then incubated in blocking buffer containing affinity-purified anti-protein VII rabbit polyclonal antibody for 100 minutes, followed by washing six times in PBS+ supplemented with 0.1% Tween 20 for 10 minutes. Cells were then incubated with goat anti-rabbit Alexa Fluor 594 (Invitrogen) secondary antibody conjugate in blocking buffer for 45 minutes, followed by washing six times in PBS + supplemented with 0.1% Tween 20 for 10 minutes, and were then washed three times in PBS + for 5 minutes. Cells were then dipped briefly in H₂O and mounted on glass slides in Vectashield mounting media with DAPI (Vector Laboratories, Inc.). DNA-binding protein (DBP) visualization was performed essentially identically using B6 mouse monoclonal IgG primary antibody (Reich *et al.*, 1983) followed by detection with goat anti-mouse Alexa Fluor 488 (Invitrogen) secondary antibody. Secondary antibody incubation was preceded and followed by washing four times in PBS+ supplemented with 0.1% Tween 20 for 10 minutes. All steps were at room temperature unless otherwise noted. Slides were sealed with nail polish and examined using a Nikon Eclipse E800 fluorescence microscope and a Princeton Instruments charged-coupled-device camera. Expression of GFP in live cells was examined using a Nikon Eclipse TE2000-E fluorescence inverted microscope and Hamamatsu ORCA-ER digital camera (Improvision Open Lab software).

2.3 Plaque assay

293 cells were plated in 6-well plates at 3×10^5 cells per well and incubated for 24 hours prior to infection. Cells were washed with PBS and infected with 1 ml of serially diluted dl309. The

assay was performed in triplicate. After 3 hours the infecting media was removed and replaced with 4 ml of agarose overlay (37.5 ml 2XMEM, 0.75 ml PenStrep, 0.225 ml yeast extract [Gibco], 0.375 ml 1M Hepes pH 7.4, 3.75 ml NCS, 0.75 ml 200 mM L-glutamine [Gibco]); mixed and warmed to 44°C before adding an equal volume of autoclaved 2% plaque agarose (Lonza) and incubated for 11 days at 37°C. After 6 days 2 ml of overlay feeder layer was added to each well (as above with the addition of 14.5 mg neutral red per 1000 ml overlay). Plaques were scored daily using light microscopy to ensure identification of all plaques.

3. Results

3.1 Equivalence of protein VII nuclear dots and infectious virus

The existence of discrete nuclear protein VII dots during early phase suggested that each dot represents an infectious viral genome. To determine if the number of dots is indeed equivalent to the number of infectious viral particles, cells were infected and monitored for protein VII dots and also for expression of the viral 72 kDa single stranded DNA binding protein (DBP), a product of the early gene E2. HeLa cells were plated on coverslips and infected with phenotypically wild-type adenovirus type 5 Δ 1309 at a low multiplicity of infection (MOI), such that significantly less than 100% of the cells were infected. For protein VII staining, the cells were fixed at 4 hours post-infection and incubated with anti-protein VII polyclonal antibody. Protein VII is the product of the late gene L2, which is expressed after the onset of viral DNA replication. Therefore at 4 hours post-infection, which is prior to viral DNA replication, all of the observed protein VII is derived from infecting viral particles and is not the result of new synthesis. This has been confirmed by the fact that incubation with hydroxyurea to prevent DNA replication does not prevent the appearance of protein VII dots in the nucleus (Chen et al., 2007; Xue et al., 2005). For DBP staining, infected cells were incubated for 24 hours in the presence of hydroxyurea to prevent viral DNA replication and subsequent reinfection, and stained with anti-DBP monoclonal antibody. The 24 hour incubation in the presence of hydroxyurea allowed for maximal DBP expression so that every cell with transcriptionally active genomes could be identified. Figure 1 shows representative staining for protein VII and for DBP.

The number of cells containing at least one nuclear protein VII dot was determined, as was the number of cells showing DBP expression. This allowed calculation of the MOI using a formula derived from the Poisson distribution:

$$\text{MOI} = -[\ln(\text{fraction uninfected})].$$

The calculated MOI along with the number of cells in each well and the dilution factor of the viral stock allowed the determination of the infectious particle concentration of the viral stock. The titer determined from measuring protein VII dots was the same as that derived from measuring DBP expression, as summarized in Table 1. This demonstrates that the number of protein VII dots in the nuclei of infected cells is equivalent to the number of transcriptionally active genomes. To further confirm the equivalence of protein VII dots with infectious particles, plaque assays were performed. As shown in Table 1 the titer calculated from plaque assay was identical to that found for DBP expression and protein VII dot quantification. These data demonstrate that quantification of protein VII dots is a very accurate measure of infectivity and plaque forming units.

A second approach was employed to relate the number of dots to the number of infectious viral genomes. In this case the number of dots within each cell was counted and this number was related to the infectious titer. HeLa cells were infected with about five times more virus than in the previous experiment to insure that virtually 100% of the cells were infected. The cells

were fixed at 4 hours post-infection and stained for protein VII. In a parallel infection cells were also stained for DBP after 24 hours to verify that virtually all of them expressed viral proteins (Figure 2). For this experiment the total number of dots in each field was determined. In addition cells were categorized based on the number of dots each of them contained. MOI was calculated as a ratio of total number of dots to the total number of cells, as presented in Table 2. From this method, the calculated infectious particle concentration was found to be nearly identical to that obtained for the experiment shown in Table 1. Moreover as shown in Table 2, the distribution of dots among different cells closely correlated with the theoretical probability distribution calculated using the Poisson distribution for the same MOI (MOI = 4.12). This indicates that protein VII dots randomly distribute among the cells in a non-cooperative fashion, as would be expected if each of them represents a single infectious genome. Since these data are in agreement with the results obtained in Figure 1 and Table 1, they confirm that each protein VII dot represents an infectious viral genome invading the host cell nucleus.

In addition to the experiments presented above, similar assays were conducted using the Adenovirus Type 5 Reference Material (ARM) obtained from the American Type Culture Collection (ATCC) (Hutchins, 2002). The results, presented in Table 3 and Figure 3, again demonstrate equivalence between the titer derived from protein VII staining and that derived from plaque assay.

3.2 Titration of helper-dependent adenovirus vector

In addition to using wild-type adenovirus, a helper-dependent (HD) adenovirus vector was also analyzed for the relationship between protein VII dots and infectious particles. This was done to test the hypothesis that a vector preparation that was created using “helper” protein VII could be used to determine accurately the concentration of infectious particles in the stock. HD vector gAd.HSU/GFP contains the GFP gene under control of the CMV promoter but is devoid of adenovirus genes. The remainder of the genome consists of human-derived intronic stuffer DNA and the adenovirus inverted terminal repeats (ITR), which mediate initiation of DNA replication (Sandig *et al.*, 2000). A schematic representation of gAd.HSU/GFP is shown in Figure 4. Although gAd.HSU/GFP does not contain any adenoviral genes, its genome is packaged in complex with protein VII due to the presence of helper adenovirus, which provides protein VII and other structural proteins (Chen *et al.*, 2007; Sandig *et al.*, 2000). During replication the helper virus genome is discarded by a crelox mechanism that rids it of the DNA packaging sequence but leaves the gAd.HSU/GFP genome intact (Chen *et al.*, 1996; Parks *et al.*, 1996). To determine how protein VII dots correlate with GFP expression for this vector, HeLa cells were infected with gAd.HSU/GFP such that significantly fewer than 100% of the cells were infected. The cells were fixed at 4 hours post-infection and stained with anti-protein VII antibody, or allowed to incubate for 24 hours for the determination of GFP expression. To maximize GFP expression cells were incubated in the presence of 50 ng/ml PMA and 200 μ M forskolin, which stimulate transcription from the CMV promoter. Additionally, 10 mM hydroxyurea was added to prevent cell replication, which would otherwise lead to a reduction in viral genomes per cell. Representative images of cells stained for protein VII or expressing GFP are shown in Figure 5. After imaging, cells were counted and average infectious particle concentrations were calculated identically as described for the experiment shown in Table 1. Table 4 shows a summary of six independent experiments, and Figure 6 shows a histogram of the results. The results for average infectious particle concentration of gAd.HSU/GFP stock obtained from either protein VII or GFP detection are indistinguishable and statistically significant, again demonstrating that observed and counted protein VII dots are numerically identical to transcriptionally active viral genomes invading the host cell nucleus.

4. Discussion

In this report it has been demonstrated that the average number of protein VII dots per nucleus of an adenovirus-infected cell population is equivalent to the average number of infectious particles per cell in that population. From a biological point of view this is important because it demonstrates that each nuclear dot represents an active infectious particle, and adds to a model in which protein VII plays an ongoing role in viral gene expression throughout the early phase of infection. It has also been shown that this information can be used to calculate directly the infectious titer of the viral stock, and that this number is equivalent to that obtained by plaque assay (Table 1). Since protein VII is an integral component of all adenovirus vectors, including helper-dependent vectors, this constitutes a general method for titration of any adenovirus stock. A second advantage is that the method can be performed in one day, compared to the 7 – 10 days or longer required for the plaque assay (Tollefson et al., 2007).

In Table 2 it is shown that the percentage of cells with a given distribution of dots fits with the Poisson distribution. However there are two features that suggest a possible biphasic distribution: the percentage of cells showing zero VII dots is higher than expected, and there are lower than expected values at 4 and 5 dots per cell. This may indicate that a proportion of the virus present in the inoculum was in small aggregates.

The assay is performed as described in Materials and Methods. A four hour infection is followed by cold methanol fixation and staining with fluorescent conjugated antibody. Fluorescence microscopy can be performed on the same day as the infection. To obtain a working titer from a previously untitered preparation it is recommended to infect with a series of dilutions of the viral stock, in duplicate or triplicate. After staining, brief microscopic observation can be used to determine which dilution produced infections where approximately 50% of the cells are uninfected. The percentage of uninfected cells can then be used directly to calculate the titer as described in Results. Alternatively, infections producing cells exhibiting dots in the range of 3 – 20 dots per cell, can be achieved for convenient counting of individual dots. The distribution of dots across the cell population on a cover slip is relatively uniform. Therefore to obtain an initial titer a single field of cells can be photographed at 60X magnification and counted. The average number of dots per cell can be converted to infectious units as described in Results and Table 2. It is suggested that for more accurate determination of titer additional fields can be counted, however it has not been observed that this affects the titer more than 33%.

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Figure 1.

Protein VII and DBP staining of low MOI infection. Cells were infected with wild-type dl309 for 4 hours and stained for protein VII, or were infected for 24 hours in the presence of hydroxyurea and stained for DBP expression. Prior to mounting, the cells were counterstained with DAPI (light gray).

Figure 2.

Protein VII and DBP staining of a high MOI infection. Cells were infected with wild-type dl309 for 4 hours and stained for protein VII, or were infected for 24 hours in the presence of hydroxyurea and stained for DBP expression. Cells were counterstained with DAPI (light gray). Arrows indicate the position of uninfected cells.

Figure 3. Titers of ARM using protein VII staining and plaque assay. Data are from Table 3. Use of 293 cells or HeLa cells is indicated.

Figure 4.

Structure of gAd.HSU/GFP. CMV, human cytomegalovirus promoter; GFP, green fluorescent protein; CRE, cyclic AMP response element; NF κ B, binding site for NF κ B; AdITR, adenovirus inverted terminal repeat; stuffer DNA, human intronic DNA sequences.

Figure 5.

Protein VII staining and GFP expression of cells infected with gAd.HSU/GFP. Cells were infected for 4 hours and stained for protein VII (Protein VII and Protein VII + DAPI), or were infected for 24 hours in the presence of hydroxyurea and analyzed for GFP expression (GFP and GFP + phase). Cells were counterstained with DAPI (light gray). Phase, phase contrast microscopy.

Figure 6. Quantification of protein VII and gAd.HSU/GFP infectivity. Data are from Table 4.

Table 1

Quantification of protein VII dots, DBP expression and infectivity

	pVII		DBP		Plaque Assay		
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 3
% positive cells/number of plaques (#positive cells/#all cells)	47.36% (547/1155)	64.30% (353/549)	41.74% (389/932)	59.83% (420/702)	21	24	24
Calculated MOI	0.64	1.03	0.54	0.91	N/A	N/A	N/A
Cells per coverslip	2.975×10^5	1.775×10^5	2.975×10^5	1.775×10^5	N/A	N/A	N/A
Dilution factor	1.25×10^5	1.25×10^5	1.25×10^5	1.25×10^5	1×10^9	1×10^9	1×10^9
Infectious particle concentration of viral stock	2.386×10^{10}	2.285×10^{10}	2.009×10^{10}	2.024×10^{10}	2.1×10^{10}	2.4×10^{10}	2.4×10^{10}
Avg. infectious particle concentration (+/- SD)	$2.336 (+/- 0.071) \times 10^{10}$		$2.017 (+/- 0.011) \times 10^{10}$		$2.3 (+/- 0.17) \times 10^{10}$		

Table 3

Determination of ARM infectious particle concentration by quantification of pVII dots in 293 and HeLa cells, as well as plaque assay.

Plaque assay (293 cells)			pVII quantification (293)			pVII quantification (HeLa)		
# of dots/# of cells	N/A	N/A	26/86	24/97	23/90	18/128	19/108	13/103
Calculated MOI (dots/cell)/number of plaques	26	23	0.302	0.247	0.256	0.141	0.176	0.126
Cells per coverslip	N/A	N/A	2.33×10^5	2.33×10^5	2.33×10^5	3.55×10^5	3.55×10^5	3.55×10^5
Dilution factor	1×10^9	1×10^9	3.5×10^5	3.5×10^5	3.5×10^5	3.5×10^5	3.5×10^5	3.5×10^5
Infectious particle concentration of viral stock	2.6×10^{10}	2.3×10^{10}	2.460×10^{10}	2.013×10^{10}	2.080×10^{10}	1.747×10^{10}	2.186×10^{10}	1.568×10^{10}
Avg. infectious particle concentration (+/- SD)	$2.2 (+/- 0.4) \times 10^{10}$		$2.324 (+/- 0.342) \times 10^{10}$			$1.956 (+/- 0.356) \times 10^{10}$		

Table 4

Detection method	% positive in each experiment						Average infectious particle concentration	Standard deviation
	1	2	3	4	5	6		
pVII (4hrs) (#positive/#all cells)	17.2% (59/343)	21.8% (87/400)	21.7% (126/581)	16.2% (71/438)	12.0% (62/517)	35.7% (184/516)	1.556×10^7	$\pm 0.761 \times 10^7$
GFP (24hrs) (#positive/#all cells)	16.9% (111/656)	13.6% (47/345)	11.7% (80/685)	14.1% (34/241)	19.6% (77/392)	16.8% (78/464)	1.124×10^7	$\pm 0.579 \times 10^7$