Nucleic Acids Research, 1997, Vol. 25, No. 16 3375–3376

Quantitative DNA slot blot analysis: inhibition of DNA binding to membranes by magnesium ions

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Received May 7, 1997; Revised and Accepted June 30, 1997

ABSTRACT

Titers of wild-type and recombinant adeno-associated viruses are routinely determined by DNA slot blot analysis. The binding of viral DNA to nylon membranes was found to be inhibited by magnesium ions, which are critical components of the DNase I digestion carried out prior to slot blot analysis. Mg^{2+} ions also interfered with the adsorption of plasmid DNA to nylon and nitrocellulose membranes. These observations yield practical insights into the poorly understood mechanisms by which DNA molecules are retained on solid supports.

The observation made by Nygaard and Hall (1), that denatured DNA and complexes between DNA and RNA can be adsorbed to nitrocellulose, paved the way for development of modern molecular biological techniques that require immobilization of nucleic acids on solid supports. These techniques include Southern and Northern transfers, dot-blotting, colony hybridization and plaque-lifts. The underlying mechanism of adsorption of nucleic acids to nitrocellulose membranes remains unclear, but has been assumed to be non-covalent (2). Studies of the interactions between nucleic acids and modified celluloses, such as nitrocellulose, have revealed that molecular weight, finite macromolecular conformation, ionic forces and weaker forces of attraction all play a role (3,4). DNA is retained on nitrocellulose only in buffers of high ionic strength. This may be because increasing salt concentration correlates with decreasing electrostatic repulsion between the phosphate groups of the DNA backbone, yielding more aggregated DNA molecules that are more easily retained on the filter (5).

Nylon membranes have a greater mechanical strength, a higher binding capacity for nucleic acids, and a stronger retention of bound nucleic acids than nitrocellulose membranes. They are able to bind both native and denatured nucleic acids in buffers of low ionic strength (6). Positively charged nylon membranes provide an ionic interaction between the negatively charged phosphate groups of the nucleic acid and the positively charged groups of the membrane.

DNA slot blot analysis is used routinely to quantitate the concentrations of adeno-associated virus 2 (AAV) particles in virus stocks (7). Since virions are generated from transfected plasmid DNA, virus stocks are treated with pancreatic deoxyribonuclease (DNase I) prior to slot blot analysis to degrade plasmid DNA as well as unencapsidated virion DNA that would lead to erroneously high estimates of viral titers. We report here that binding of viral DNA released from disrupted virions to a nylon membrane is inhibited in the presence of the Ca^{2+} and Mg^{2+} ions required for DNase I activity (8). Further studies with highly purified plasmid DNA revealed that Mg^{2+} is sufficient to inhibit binding of DNA to nylon as well as nitrocellulose membranes.

AAV was generated from plasmid pSM620 (9) as described previously (7). Ten µl of the virus preparation were either not treated or treated with DNase I (100 U/ml) at 37°C for 30 min in the presence or absence of $CaCl_2$ and $MgCl_2$. Virions were disrupted to release denatured viral DNA by incubation at 65°C for 1 h in NaOH at a final concentration of 0.5 N. Following neutralization with an equal volume of 20× SSC, the samples contained final concentrations of 0.625 mM Ca^{2+} and 1.25 mM Mg^{2+}, denoted as 1×. Two-fold serial dilutions of each sample were aspirated onto a positively charged nylon membrane (GeneScreen *Plus*®, NEN® Research Products, Boston, MA) using a slot blot apparatus and probed with ^{32}P-labeled AAV DNA synthesized from the *Xba*I fragment of p*sub*201 (7) using random oligonucleotide primers. Band intensities of the hybridization signals were determined using the Bio-Rad GS-250 Molecular Imager and Densitometer.

Figure 1A shows that 1× concentration of Ca^{2+} and Mg^{2+} completely inhibited binding of AAV viral DNA to the nylon membrane. Although Ca^{2+} and Mg^{2+} were added in the form of chloride salts, the chloride anions are not responsible for inhibiting binding since the amount of chloride added in the form of $CaCl_2$ and $MgCl_2$ is 400-fold less than that present in the 10× SSC common to all samples. The presence of DNase I does not affect binding (Fig. 1).

Figure 1B shows that binding of AAV plasmid DNA, pSM620, to GeneScreen *Plus*® nylon membrane is also inhibited by Ca^{2+} and Mg^{2+}. Binding of plasmid DNA is less affected than binding of viral DNA (Fig. 1). Since approximately equal amounts of DNA were analyzed in each case, these results suggest that DNA structure (circular, double-stranded plasmid versus linear, single-stranded viral DNA) may play a role in the interaction with a positively charged nylon membrane. To test this, pSM620 was digested with *Pvu*II to excise the AAV sequences prior to denaturation in the presence or absence of varying amounts of

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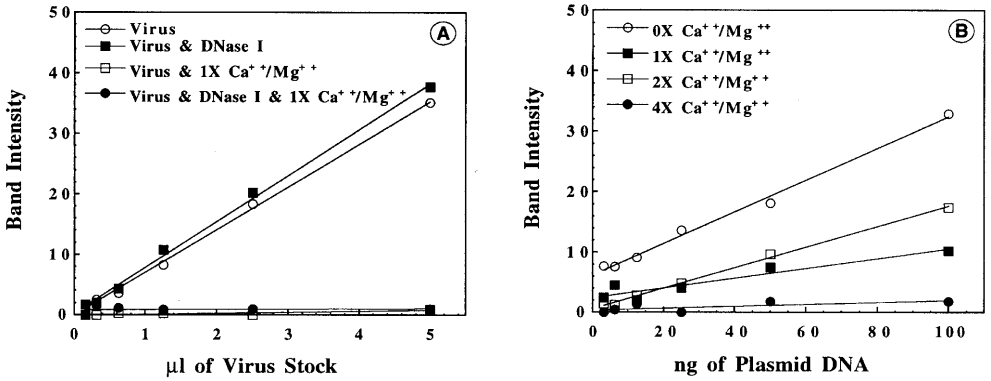

Figure 1. Binding of viral and plasmid DNA to positively charged nylon membranes is inhibited by Ca^{2+} plus Mg^{2+}. Two-fold serial dilutions of known amounts of AAV stock (**A**) or pSM620 (**B**), that were treated or not treated with DNase I (100 U/ml) in the presence or absence of the indicated amounts of Ca^{2+} and Mg^{2+}, were applied to GeneScreen *Plus*® nylon membranes using a slot blot apparatus. The membranes were baked, hybridized to the AAV probe, washed, and autoradiographed at –70°C for 90 min. The autoradiographic images were quantitated using the Bio-Rad GS-250 Molecular Imager and Densitometer. Optical density values are graphically represented.

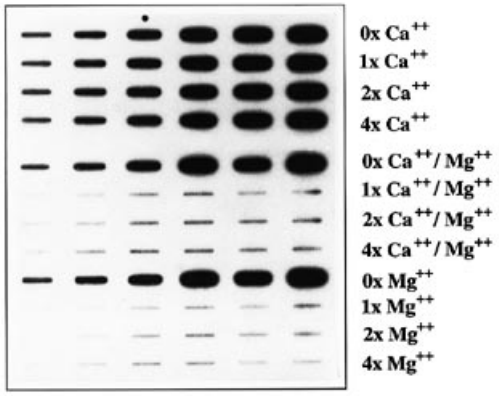

Figure 2. Mg^{2+} alone is sufficient to inhibit binding of DNA to a nylon membrane. Two-fold serial dilutions of known amounts of pSM620 in the presence or absence of the indicated amounts of Ca^{2+} and/or Mg^{2+} were analyzed as described in the legend to Figure 1.

Ca^{2+} and Mg^{2+}. Binding to GeneScreen *Plus*® nylon membrane of AAV sequences in the context of a circular plasmid was inhibited ~3-fold less by Ca^{2+} and Mg^{2+} than binding of the linearized sequences, although the ratio of cations to DNA was constant (data not shown). Thus, the interaction of DNA with nylon appears to be influenced by DNA structure which, in turn, may be affected by divalent cations.

Interestingly, Mg^{2+} increases the secondary structure of tRNA molecules which decreases their retention on derivatized DEAE–cellulose (3). It was therefore of interest to determine if Mg^{2+} alone is sufficient to inhibit binding of DNA to nylon. As shown in Figure 2, Mg^{2+} alone inhibits binding of DNA to GeneScreen *Plus*® nylon membrane as efficiently as Mg^{2+} plus Ca^{2+}. Mg^{2+} was also observed to inhibit binding of DNA to another positively charged nylon membrane (MAGNACHARGE, Micron Separations, Inc., Westborough, MA) as well as to a nitrocellulose membrane (Protran®, Schleicher & Schuell, Keene, NH) (data not shown). Based on these results, Mg^{2+} ions required in manipulations of nucleic acids need to be chelated with EDTA prior to adsorbing the nucleic acids to a solid support. This is especially noteworthy since many restriction enzyme buffers contain $MgCl_2$. We found that addition of EDTA (100 mM final concentration) prior to denaturing plasmid DNA completely restored DNA binding to nylon, even in the presence of 4× concentration of Mg^{2+} (data not shown).

The results presented here indicate that the mechanisms whereby nucleic acids are retained by nylon and nitrocellulose matrices may be similar in that both depend on ionic and non-ionic interactions. These interactions are influenced by the macromolecular conformation of the nucleic acid which appears to be affected by Mg^{2+} ions. To ensure accurate quantitation of DNA by slot blot analysis, a DNA standard with a similar macromolecular structure to that of the unknown should be used.

ACKNOWLEDGEMENTS

We thank Dr Richard C.Bockrath for a critical review of this manuscript. This research was supported by grants from the National Institutes of Health (HL-48342, HL-53586 and DK-49218, Centers of Excellence in Molecular Hematology), and Phi Beta Psi Sorority. A.S. was supported by an Established Investigator Award from the American Heart Association.

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