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Determination of the molecular weight of DNA-bound protein(s) responsible for gel electrophoretic mobility shift of linear DNA fragments examplified with purified viral *myb* protein

Hilmar Bading

Max-Planck-Institut für Molekulare Genetik, Abt.Schuster, Ihnestrasse 73, 1000 Berlin 33, FRG

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ABSTRACT

A protein-DNA complex has less gel electrophoretic mobility than the free DNA fragment. One parameter for the degree of retardation of a linear DNA fragment in a protein-DNA complex is the molecular weight of the bound protein(s). The quotient of the migration distances of free DNA (m) and protein-DNA complex (m') is a function of the molecular weight (MW) of the bound protein(s). Based on the evaluation of the lac repressor induced mobility shift of a 203 bp DNA fragment containing the lac operator in a 5% non-denaturating polyacrylamide gel a direct proportionality could be shown between (m/m'-1) and MW with the proportionality factor K = 215 kDa. The factor K depends on the acrylamide concentration in the gel, getting lower values with increasing acrylamide concentrations. A calculation is given to determine the molecular weight of DNA-binding factors responsible for the decreased electrophoretic mobility of a linear DNA fragment. As an example this calculation was used in order to analyse DNA-binding of the isolated viral myb protein. It could be demonstrated that the viral myb protein binds to DNA as a monomer and as a dimer.

INTRODUCTION

Electrophoretic mobility shift assay has become an important assay for analysing protein-DNA interaction. The assay is based on the altered mobility of DNA in a protein-DNA complex during non-denaturating polyacrylamide gel electrophoresis. The complexes are resolved as discrete bands with mobilities decreasing as a function of the molecular weight of the participating protein(s). The assay has been used in equilibrium and kinetic analysis of purified prokaryotic gene regulatory proteins with known molecular weight (1). More recently, crude nuclear extracts from mammalian cells have been used in the mobility shift assay for analysing sequence specific DNA-binding properties of putative eukaryotic gene regulating factors (2). The viral myb (v-myb) gene of the avian myeloblastosis virus (AMV) codes for a nuclear oncogene product, which is responsible for the tumorigenic properties of AMV (3,4,5). The v-myb protein is known to bind to DNA in vitro and is supposed to be involved in gene regulation (5,6,7,10). In this study the DNA-binding properties of the immunoaffinity purified v-myb protein were analysed by the use of gel electrophoretic mobility shift assay and its evaluation using the method for calculating the molecular weight of the protein(s) participating in a protein-DNA complex.

MATERIALS AND METHODS

Immunoaffinity purification of v-myb protein

The v-myb protein was isolated by immunoaffinity chromatography using lysates of the metabolically labeled chicken cell line BM-2 as described (5,8).

Electrophoretic mobility shift assay

Protein-DNA complexes were formed by addition of 15-30 ng of immunoaffinity purified v-myb protein to binding buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT) containing 1 ng of the 81 bp SphI/HinfI lambda DNA fragment (position 23947-24027, ref.8), which has been end-labeled in vitro using T4 DNA polymerase and [α-^{32}P]dATP. As competitor DNAs the alternating co-polymer duplices poly[d(I-C)] or poly[d(A-T)] (Boehringer Mannheim, West Germany) were used, which were added to the reaction mixture at the same time the labeled lambda DNA fragment was added. After 30 min incubation at 30°C, the resulting complexes were resolved in polyacrylamide gels (4%, 5%, 6%, 7%, 8%, 9%, or 10% acrylamide; acrylamide: bisacrylamide weight ratio of 30:1) containing 0.5x TBE-buffer (1x TBE, 89 mM Tris-borate, pH 8.0, 2 mM EDTA). Protein-DNA complex formation in the presence of antibodies was performed by preincubation of 15 ng purified v-myb protein either with v-myb specific monoclonal antibodies (8) or control antibodies for 30 min at 30°C in binding buffer without DTT and supplemented with 1mg/ml bovine serum albumine, followed by incubation for 30 min at 30°C with the labeled DNA and electrophoresis on a 5% polyacrylamide gel. The gels were pre-run for 1 h in 0.5x TBE-buffer at a voltage gradient of 12 Vcm^{-1}. When all samples had been loaded electrophoresis was carried out at 6 Vcm^{-1} in 0.5x TBE-buffer. After electrophoresis the gels were soaked for 10 min in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 5% glycerin, dried, and autoradiographed at -70°C. Electrophoretic mobility shift assays with 40 units of T7 RNA polymerase (Boehringer Mannheim, West Germany) and 1ng of the end-labeled SphI/HindIII DNA fragment from pT712 (Bethesda Research Laboratories, Bethesda, MD) were performed in 5% polyacrylamide gels as described for the v-myb protein.

RESULTS

During a non-denaturating polyacrylamide gel electrophoresis with the electric field strength E, the migration distance m of a linear DNA fragment with ion mobility μ is a linear function of time t. It follows the equations

$$dm/dt = \mu E$$

$$m = \mu Et \quad (1)$$

A protein-DNA complex has a reduced ion mobility μ', which appears as a decreased electrophoretic migration distance m'. μ' depends on the ion mobility of the DNA fragment

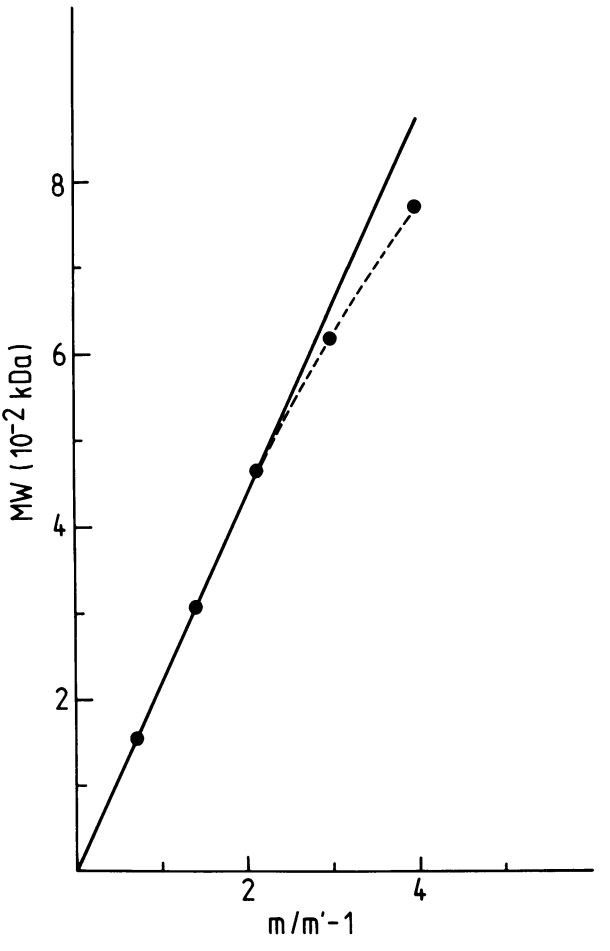

Figure 1: Plot of the molecular weight of lac repressor proteins against the quotient of the migration distance (m) of the free 203 bp lac operator containing DNA fragment and lac repressor induced decreased migration distance (m') minus 1. The dotted line indicates the deviation from the linear relationship in the high molecular weight range.

(μ), and on the charge, structure, and the molecular weight of the DNA-bound protein(s). On the assumption, that μ' is infinitesimally influenced by the protein structure, the protein(s) do not exhibit DNA-bending properties, and the charge of the protein(s) are negligible compared to the charge of the DNA under the assay conditions, μ' solely depends on μ and is a function of the molecular weight (MW) of the DNA-bound protein(s).

(2) $$\mu' = \mu\ f(MW)$$

m' may be expressed in terms of μ and f(MW) applying equations 1 and 2.

$$m' = \mu' Et$$

(3) $$m' = \mu\ Et\ f(MW)$$

The quotient m/m' eliminates the term μEt and yields m/m' as a function g(MW) of the molecular weight of the protein (MW).

(4) $$m/m' = 1/f(MW) = g(MW)$$

The recently published lac repressor induced gel electrophoretic mobility shift of a 203 bp DNA fragment containing the lac operator sequence in a 5% polyacrylamide gel yields several discrete bands with decreased mobilities, representing lac repressor-DNA complexes. The degree of retardation depends on the quantities of lac repressor tetramers bound to the DNA (1). These data were evaluated for plotting the relationship between MW of the protein component of the complexes and m/m'. The graph is linear in a wide range of MW (0-450 kDa) and curves in the high molecular weight range. Since in the case of absence of protein m/m'= 1, the molecular weight of the protein(s) participating in a protein-DNA complex is directly proportional to m/m'-1 with the proportionality factor K, which is K = 215 kDa for a 5% acrylamide gel concentration (Figure 1).

(5) $$MW = (m/m' - 1)\ K$$

When 5% polyacrylamide gels were used for analysing protein induced electrophoretic mobility shifts of linear DNA fragments, the proportionality factor K = 215 kDa could be applied together with the measured values for m and m' to equation 5 in order to determine the approximate molecular weight of the DNA-bound protein(s).

Recently, a selective DNA-binding of the purified human c-myb protein, the cellular homologue of the v-myb protein, to a lambda DNA fragment has been described (10). This lambda DNA fragment contains a 81 bp SphI/HinfI subfragment, which was used in this study to analyse the DNA-binding properties of the immunoaffinity purified v-myb protein. Figure 2A shows a v-myb protein induced mobility shift of the 81 bp DNA fragment in a 5% polyacrylamide gel. Beside the free DNA, two additional discrete bands with decreased mobilities are resolved in the gel, representing two distinct protein-DNA complexes indicated as complex 1 and complex 2. Previous work has shown that specific antibodies can either disrupt or further retard protein-DNA complexes in non-denaturating gels (11). The addition of v-myb specific monoclonal antibodies (8) to the binding reaction prevents formation of

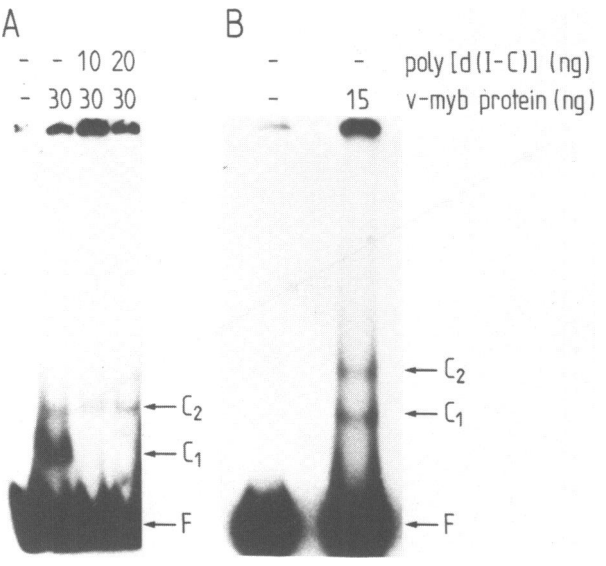

Figure 2: Gel electrophoretic mobility shift of the 81 bp DNA fragment (1 ng) caused by the purified v-myb protein in a 5% (A) and 7% (B) polyacrylamide gel in the presence of indicated amounts of poly[d(I-C)]. F indicates the free DNA, C_1 and C_2 represent the protein-DNA complexes 1 and 2.

the two complexes, whereas the complexes persist in the presence of a control monoclonal antibody against the replicase gene product of the bacteriophage MS2 (data not shown). This demonstrates that v-myb protein is indeed responsible for the two complexes. Since the v-myb protein alone fails to enter the non-denaturating gel under the assay conditions (data not shown), the protein charge is small compared to the DNA fragment. Using equation 5, the calculated molecular weights of the DNA-bound proteins are 48 kDa for complex 1 and 97 kDa for complex 2. Since the molecular weight of the v-myb protein is 48 kDa (5,8), the two bands represent complexes with one and two v-myb protein molecules, respectively, bound to the DNA fragment. In complex 2 the DNA-binding of the v-myb proteins takes place as either two monomeric forms to two different DNA-binding sites or one dimer to only one DNA-binding site. However, when poly[d(I-C)] was used in competition experiments, the DNA-binding of the v-myb monomer in complex 1 disappeared and only complex 2 persisted (Figure 2A). This demonstrates that the v-myb monomer exhibit only a weak DNA-binding to the 81 bp lambda DNA fragment, whereas the more stable complex 2 is formed by a v-myb dimer. This might indicate a cooperative effect. Furthermore, complex 2 remains stable up to 500 ng of competing poly[d(I-C)] or poly[d(A-T)], demonstrating a strong DNA-binding of the dimeric form of v-myb protein to the 81 bp

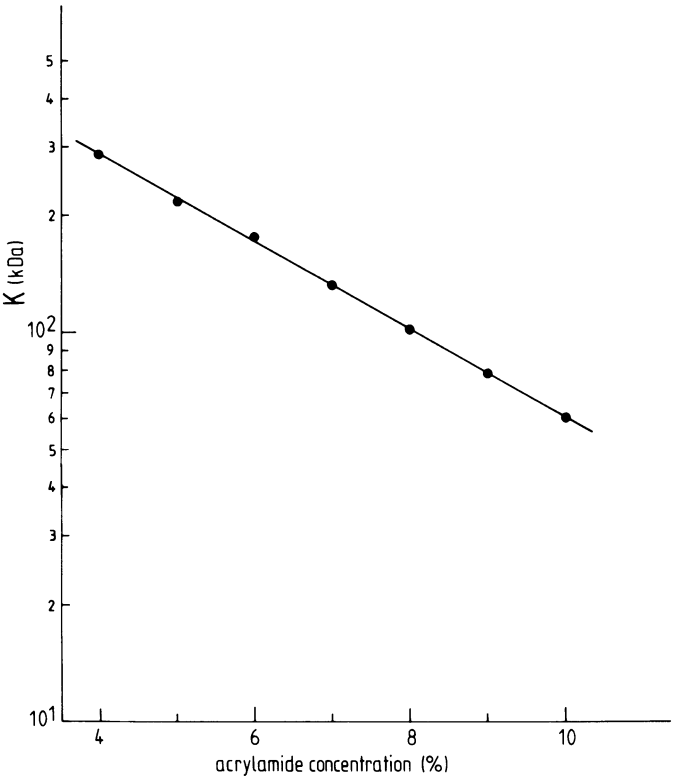

Figure 3: Semilogarithmic plot of acrylamide concentrations of gels used for electrophoretic mobility shift assays against proportionality factor K of the protein molecular weight calculation.

DNA fragment with at least 500 fold higher binding affinity compared to unspecific DNA (data not shown). On the assumption, that the 81 bp DNA fragment contains one binding site for the v-myb protein, the molar ratio of protein to DNA-binding sites is about 30:1 under the used assay conditions. Since about 1-10% of the DNA applied to the assay exhibit a retarded electrophoretic mobility, less than 1% of the purified v-myb protein is able to form a stable protein-DNA complex in this assay.

Beside the influence of the molecular weight of DNA-binding protein(s), the intensity of retardation of a linear DNA fragment during electrophoresis also depends on the acrylamide concentration in the gel. Figure 2B shows a v-myb protein induced electrophoretic mobility shift of the 81 bp DNA fragment in a 7% polyacrylamide gel. The complexes 1 and 2 exhibit less mobility in the 7% gel compared to the 5% gel shown in Figure 2A, indicating that higher acrylamide concentrations lead to an amplified retardation

of a protein-DNA complex. Hence, the proportionality factor K in equation 5, which is used for calculating the molecular weight of DNA-bound protein(s), also depends on the acrylamide concentration in the gel. In order to determine this dependence, the v-myb induced mobility shift of the 81 bp DNA fragment was analysed in gels with various acrylamide concentrations. Since the molecular weights of the DNA-bound proteins in complex 1 and 2 are known, the proportionality factor K could be evaluated for each acrylamide concentration by the use of equation 5. The data can be made to fit a straight line by plotting log K against the acrylamide concentration (Figure 3).

To prove the validity of the calculation method for other DNA-binding proteins gel electrophoretic mobility shift assays were performed with T7 RNA polymerase, a single polypeptide with 98kDa molecular weight, and a DNA fragment containing the T7 promotor. In the presence of 0.5-1.0μg of competing poly[d(I-C)] a protein-DNA complex is formed, which yields a calculated molecular weight of 90 kDa for the participating protein (data not shown). The calculated molecular weight of the protein involved in a second protein-DNA complex, which appears to less extend mainly in the absence of poly[d(I-C)], amounts to 186 kDa and likely represents 2 molecules of T7 RNA polymerase bound to the DNA fragment (data not shown). Evaluation of BspRI methylase DNA mobility shift data, which were kindly provided by A. Schulz and M. Noyer-Weidner, yields a calculated molecular weight of 49 kDa for the protein component of the formed protein-DNA complex, which corresponds well with the known BspRI methylase molecular weight of 50 kDa.

DISCUSSION

The method described here allows the molecular weight determination of DNA-binding protein(s), which form a protein-DNA complex and are responsible for a decreased gel electrophoretic mobility of linear DNA. The presented calculation is valid provided that under the assay conditions the charge of the protein(s) is negligible compared to the charge of the DNA fragment. Carey had shown previously that the trp repressor induced DNA mobility shift depends on the charge of the protein (12). At pH 8.3 the trp repressor provides little retardation effect, because the protein has a negative charge and its electrophoretic mobility is very similar to that of the DNA (12). DNA-binding proteins, which alone exhibit a mobility in the non-denaturating gel similar to that of the DNA do not fulfill the condition for the calculation method. Furthermore, the degree of protein induced DNA retardation might depend on the lenght of the DNA fragment dealt with. However, the DNA length for determining the proportionalality factor K in a 5% polyacrylamide gel was 203 bp (Figure 1), and this factor proved useful in evaluating the v-myb induced mobility shift of a 81 bp DNA fragment. Hence, the calculation has validity at least for linear DNA fragments with lengths between 81 bp and 203 bp. A second assumption for the molecular weight determination is the use of linear DNA fragments. Non-linear but bent DNA fragments exhibit a less gel electrophoretic mobility than linear DNA fragments of the same size. DNA-

binding proteins that cause DNA bending are responsible for an additional bending induced retardation of a DNA fragment during electrophoresis, which leads to a wrongly high calculated molecular weight of the bound protein(s). On the other hand a more decreased mobility of a DNA fragment than it would be expected from the known molecular weight of the bound protein(s) might be interpreted as an ability of the protein(s) to bend DNA.

Beside the correctness of the acrylamide concentration in the gel the measurement of the mobilities, which were determined to an accuracy of 1-2%, mainly accounts for the error of the calculation method. Provided that the molecular weight of the DNA-binding protein(s) is in the linear range of the relationship between MW and m/m'-1, i. e. 0-450 kDa for 5% polyacrylamide gels (see Figure 1), the error of the calculation method amounts to about 10%.

The presented molecular weight determination method proved useful in analysing the DNA-binding properties of the purified v-myb protein. It could be demonstrated, that the two v-myb-DNA complexes carry the v-myb monomer and the v-myb dimer, respectively. It is possible, that the calculation method can complement alternative procedures such as UV cross-linking of protein-DNA complexes and helps analysing other DNA-binding proteins.

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