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Detection of Protein-DNA Binding in Crude Nuclear Extract Using Biacore Assay

Zhong-Lian Li[1]*, Kenta Nagahori[1], Shinichi Kawata[1], Yuki Ogawa[1], Hidenobu Miyaso[1], Ning Qu[1], Shogo Hayashi[1], Masahiro Itoh[1] and Yoshinori Otsuki[2]

[1]*Department of Anatomy, Tokyo Medical University 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan*

[2]*Department of Anatomy and Biology, Osaka Medical University 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan*

Abstract

Biacore is a system that is extensively used to characterize the interactions between molecules in terms of their binding specificity, affinity, and kinetics. The practical procedures, however, for measurement of protein-DNA association in crude nuclear extract are yet to be defined. In the present study, DNA fragments with the least protein binding activity were identified in database for transcription factors and included in Biacore assay as control, so that the signals from non-specific binding were markedly suppressed. It was known that when analytes were purified transcription factors, the dissociation curves in Biacore sensorgrams showed exponential tendency. Further analysis showed that the interaction between ERα complex from crude nuclear extract and DNA oligos could be fitted to mono- or bi-exponential functions. Discrimination between orders of exponential function was based on the results of several statistical analyses with an average score of more than 95%. As exponential characteristics allow extrapolation of the dissociation, theoretical amount of bound anti-ERα antibodies could thus be evaluated statistically. Our procedures made Biacore a practical technique like Supershift Assay to measure protein-DNA association in crude nuclear extract with reproducible and reliable results.

Keywords: Biacore; Exponentiation; Extrapolation; Estrogen receptor; Surface plasmon resonance; Crude nuclear extract

Introduction

It is well known that association of transcription factors to DNA is one of the crucial steps that initiate transcription of genes. Protein-DNA or protein-RNA interactions are usually analyzed *in vitro* using such techniques as electrophoretic mobility shift assay (EMSA) [1,2] and/or DNase footprinting [3]. When an antibody is used to identify a protein present in the protein-DNA complex in EMSA, the method is usually referred to as Supershift Assay. Ample knowledge concerning the specificity, affinity, and kinetics of protein-DNA association are brought to light with these techniques. Nowadays, chromatin immunoprecipitation (ChIP) [4] has been employed in large-scale genomic research of protein-DNA interaction [5]. All these techniques, however, can reveal a point of DNA-protein binding, rather than a real time association and dissociation as Biacore can exhibit [6].

Based on the surface plasmon resonance (SPR) mechanism, the well-known Biacore technology allows measurement of change in mass concentration on metal surface as molecules associate or dissociates [7]. The technique is well suited to determination of kinetic parameters, and both kinetic and affinity constants can be derived from the sensorgram data. An important chip made available by Biacore is the SA-Sensor Chip, which can be used to measure protein-DNA interaction. This chip was applied for measuring the kinetic constants of the interaction of the DNA-binding domain of hepatocyte nuclear factors (HNF)-3 alpha with its transferrin enhancer DNA specific target site [8]. In another study, it was used to analyze the difference in affinity for DNA between high mobility group (HMG) proteins 1 and 2 [9]. It is widely accepted that more information can be obtained by use of Biacore than that of the corresponding conventional techniques. Although other machines based on SPR have recently emerged in addition to the most recent Biacore model, it is noteworthy that only bindings between purified protein molecule and DNA oligo were analyzed in most of these studies [7]. As for statistical analysis of association and dissociation between purified analyte and ligand, curve regression method, such as Langmuir Model, has been adopted [6]. To the best of our knowledge, however, statistical method has yet to be fully established for analysis of the association between a complex of molecules from crude nuclear extract and DNA oligos with protein-specific binding motifs.

Presently, there are only several reports concerning the protein-DNA associations in crude nuclear extract using SPR technique [7,10]. Due to the strong electrostatic nature of SPR, these authors have explored several new approaches, including a new equation in order to optimize experimental conditions for reproducible results. Despite of their efforts, a question remains as to how to exhibit the non-specific binding using a reasonable control. Another question is that when using an antibody to identify one of the associated molecules as in Supershift Assay, the significant association for the bound protein complex and/or corresponding antibodies is yet to be defined statistically [11].

In the present study, DNA fragments with the least protein binding activity were identified in transcription factor database and included as control in Biacore analysis, so that the signals from non-specific binding were markedly suppressed. Further experiments showed that the dissociation could be expressed mono- or bi-exponentially for the bound ERα complex from crude nuclear extract. The exponential characteristics made it possible to extrapolate the values of bound ERα complex after the inflow of anti-ERα antibodies. The significant

***Corresponding author:** Zhong-Lian Li, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan, Tel: +81-3-3351-6141, Ext: 231; Fax: +81-3-3341-1137; E-mail: zhonglan@tokyo-med.ac.jp

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association could then be defined by comparing the amount of the bound antibodies between the experimental and control. Our procedures made Biacore a practical technique to measure protein-DNA association in crude nuclear extract, like a Supershift Assay on chip with reproducible and reliable results.

Materials and Methods

DNA sequences with the least protein binding activity

It is suggested that several databases containing most, if not all, of the transcription factors for mouse or human have been established, which indicates the feasibility, albeit theoretical possibility, to search for the DNA sequences that do not specifically bind to any known transcription factors. The search was carried out against TransFac Public database for *homo sapiens* [12]. For all possible combination of four DNA bases up to 48 pairs in length (4^{48}), the sequences with the cutting score ≤ 75 (Figure 1) were reserved for further theoretical analysis. One of the reserved sequences (NoMf-32) was included in DNA-protein binding experiments (Table 1).

Preparation and immobilization of DNA Oligos

The bottom strand sequence information for estrogen responsive element ERE (Table 1) in *Xenopus* vitellogenin A2 gene (No. X00205.1) was obtained from GenBank (NIH, Bethesda, MD) and arbitrarily named (EREw32). Its mutant (EREm32) was generated using TransFac Public. Biotinylated and purified DNA oligo fragments were obtained from Invitrogen (Carlsbad, CA). Only the 3` ends of the bottom strand were biotinylated. To make double-stranded oligos, the non-biotinylated top strand and the biotinylated bottom strand were added at a molecular ratio of 3:1 to a standard PCR buffer containing 25 mM $MgCl_2$. Then the oligos were annealed by heating at 98°C for 10 min, cooling to 50°C at a rate of about 2°C /min, and then chilling on ice. The double strands generated in this way could be subcloned and sequenced (data not shown).

Biacore assay

Protein-DNA binding experiments were conducted as reported previously [13-15], using the Biacore 2000 system, Sensor Chip SA, and HBS buffer (pH 7.4) containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (from Biacore AB, Uppsala, Sweden). To immobilize the biotinylated DNA fragments to the sensor chip, 0.1 μM of the annealed oligos in HBS buffer containing 150 mM NaCl was injected at a rate of 5 μl/min over one of the four flow cell of SA Sensor Chip. The first flow cell was left vacant; the second flow cell was immobilized with the DNA oligos without known protein-specific binding motifs. The remaining two were kept for oligos containing ERE motifs and their respective mutants. The average amount immobilized was 350 ± 5 RU for each type of oligos.

Oligos	Motif sequences	Consensus (%)
NoMf-32	5`-act cgg tcc gag cgt agc cta gtc cta ggc ta	<75%
EREm32	5`-ttg atc aag gcc ctg gga cca gac ttt ctc ga	<75%
EREw32	5`-ttg atc agg tca ctg tga cct gac ttt ctc ga	100%

Table 1: Estrogen Responsive Element (ERE) containing oligos.

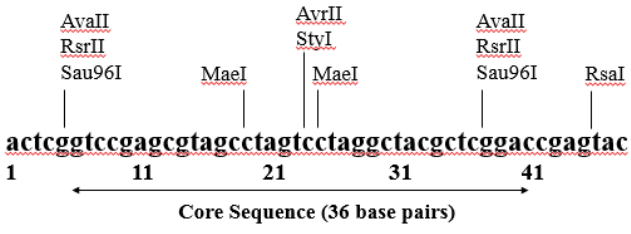

Figure 1: Characteristics of one of the 108 sequences with the cutting score ≤ 75 and a core sequence of 36 base pairs in length. Some of the sites for restriction enzyme were indicated.

HBS buffer with 25 ng/μl poly [d (I-C)] was used to prepare the nuclear proteins for experiments. Human recombinant ERα (Promega Biosciences; Madison WI) was injected at a concentration of 8 footprinting units/ml, corresponding to 0.005 μg/μl of nuclear protein. A final concentration of 0.10, 0.15, or 0.25 μg/μl was used for crude nuclear extract from MCF-7 human cancer cells (Santa Cru Biotechnology, Dallas, TX). The samples were incubated at 24°C for 15 min. All anti-ER antibodies were from Santa Cruz Biotechnology. Each association/dissociation cycle was performed at a constant flow rate of 30 μl/min. For DNA-protein binding reactions, each sample was injected over the four flow cells for 5 min, followed by a constant flow of HBS buffer for 5 min to observe dissociation of bound proteins. Then 0.02 μg/μl of an antibody in HBS buffer was injected in the same manner. At the end of each cycle, bound proteins were removed by two-pulse injections of 1.5 M NaCl containing 0.05% of surfactant P20 for 30 secs each to regenerate the chip. Sensorgrams were recorded automatically and adjusted to zero baseline level by subtracting the response recorded immediately before the injection of each sample. Each measurement was repeated three times with identical results. Comparison between sensorgrams was carried out by subtracting the responses from the first or the second flow cell.

Extrapolation and analysis of sensorgrams

Implication of exponential regression: Although it is not known exactly how many molecules in crude nuclear extract bind to DNA oligos, the bound molecules could be considered as a complex of molecules in dissociation phase. Therefore, statistical method based on exponential Langmuir Modes for purified analyte [16] could be tentatively applied to our data, and the implication of suitable functions might arise from trying to fit the bound proteins in dissociation phase to a linear equation:

$$Y_i = a + b \times T_i$$

where Y_i are the bound proteins at time T_i, and a and b are parameters, after transformation of values of either the time (T_i) or bound proteins (Y_i) or both to their natural logarithms, square roots, squares, reciprocals, or exponents. This process was mainly carried out using JMP 5.1 (SAS Institute, Cary, NC, USA). Suitable functions approximate to the dissociation values were assumed to abide by three conditions in extrapolation. First, the extrapolated values of bound nuclear proteins beyond 900 seconds should be lower than that of the antibodies. This is because the dissociation rate of antibodies is generally very slow. In addition, values of bound antibodies in dissociation phase should always be higher than the extrapolated values for bound nuclear protein. And thirdly, these values at any time points should approximate zero gradually. Results from this try-and-error process showed that the exponential regression was still applicable to crude nuclear extract.

Determination of exponential order and function: Presently, it is considered that there are no standard criteria for discrimination of the goodness-of-fit in terms of the exponential order. Because of its automatic multiple disciplines concerning discrimination,

W3DIMSUM 1.5 Beta [17], an online Java application at its default setting was employed for regression and determination of the exponential order:

$$Y(T)=\sum_{i=1}^{n} A_i e^{(LiT)}$$

where Y (T) is the expected value of the data at time T, n is the order of the model, A_i are initial value, and L_i is the kinetic parameter of dissociation constant (Kd) for bound molecule complex. Due to various limitations of W3DIMSUM, about twenty data points of bound nuclear proteins for regression were extracted at an interval of 15 seconds out of the dissociation data from 300 to 900 seconds. Extrapolated values for nuclear proteins in dissociation phase were calculated (JMP 5.1) using equations from W3DIMSUM. In order to estimate the discrepancy that might result from the data extraction, the extrapolated values and actual data of dissociation were fitted to mono- or bi-exponential function:

$$Y = A_1 \times e^{(Kd1T)} + A_2 \times e^{(Kd2T)}$$

and then compared using ANOVA table of linear fitting for Degrees of Freedom. In addition, Reduced Chi-squared (Chi-Sqr) test, Adjusted (Adj.) R-Square test, and Residual Plot Analysis were employed for goodness-of-fit. There processes were carried out mainly using OriginLab 7.5 (OriginLab Corporation, Northampton, MA, USA).

Extrapolation of bound nuclear proteins and antibodies: The area-under-curve (AUC) of from 1000 to 1200 seconds was presented as the sum of bound antibodies and nuclear proteins (AUCan) in the dissociation phase of antibodies. It was calculated using OriginLab 7.5 based on the trapezoidal rule [18]:

$$Y = Y0 + A1 \times e^{(-T/Kd)} + A2 \times e^{(-T/Kd)}$$

where Y_i is bound proteins at time T_i of up to n data points, and values of the next time point are Y_{i+1} and X_{i+1}. Theoretical AUC of the bound nuclear proteins (AUCnp) in this phase was calculated in the same way, after extrapolation of the values using equations from W3DIMSUM. The amount of bound antibody (AUCab) in its issociation phase was obtained by subtraction of the extrapolated AUCnp from AUCan.

Results

Artificial DNA sequences with the least protein binding activity

Searching all possible combination of four DNA bases up to 48 pairs in length (4^{48}) against TransFac Public database for all organisms, 108 sequences with the cutting score ≤ 75 (Figure 1) were identified. These sequences exhibit a core sequence of 36 base pairs in length, and can be recognized by such restriction enzymes as AvrII, StyI, MaeI, and others. The cutting score (Figure 2) was as low as 52 for a sequence of 8 base pairs in length. Then the cutting score reaches a plateau of 75 for sequences between 33 and 48 base pairs. Further searching the core sequence against the databases [19] of NBCI Genomes and human and mouse genomic plus transcript with BLASTN 2.6.0+ [20] showed that no significant similarity was found, which indicate that the presented sequences are artificial. The protein binding activities for one of these sequences were then tested in the following experiments (Table 1).

Lowest binding values of DNA oligos with the least protein binding activity

Following association of either recombinant ERα or nuclear extract from MCF-7 cells, while responses from NoMf-32 oligos with the least protein binding activity could always be detected (Figures 3a, 3c, 4a, and 4c), they were always lower than that of mutant (EREm32) and wide type ERE (EREw32). For recombinant ERα, while the highest value 67.45 RU was detected on EREw32, 40.23 RU was shown on EREm32, but only 15.82 RU was exhibited on NoMf-32. For crude nuclear protein, while the highest value 216.67 RU was detected on EREw32, 200.32 RU was shown on EREm32, but only 166.35 RU was exhibited on NoMf-32. It should be noted that the bound molecules from crude nuclear extract dissociate faster from NoMf-32 and EREm32 than that from EREw32. The results indicate low affinity of the DNA oligos with the least protein binding activity. The responses differ markedly following inflow of antibodies. After injection of anti-ERα antibody, no obvious responses could be observed in NoMF-32 or EREm32 (Figure 3a). However, a slightly uprising curve for EREm32 was exhibited after subtraction of NoMf-32 (Figure 3b). Similar responses were also shown in the dissociation phase of crude nuclear extract (Figures 3c and d), indicating the highest rise in sensorgrams for EREw32 was always observed for recombinant ERα and nuclear extract (Figures 3 and 4). It should be noted that there was an acute and sharp rise in sensorgram when inflow of antibody against phosphorylated (S106) ERα (Figure 4) was initiated, and then a drastic decrease was exhibited in dissociation phase. For recombinant ERα (Figure 4a), while the highest value of 269.67 RU was exhibited on EREw32, 204.58 RU was shown on EREm32, but even 183.33 RU was detected on NoMf-32. For crude nuclear protein (Figure 4c), while the highest value of 296.37 RU was recorded on EREw32, 252.52 RU was reflected on EREm32, but 233.35 RU was still detected on NoMf-32. One of the reasons might be that a large amount of phosphorylated ERα and its co-factors could be loosely associated either specifically or non-specifically. All these results indicate that using vacant flow cell as a control might lead to deviation in evaluation of DNA motif-specific bindings.

Extrapolation of sensorgrams for crude nuclear protein

To extrapolate the dissociation values after injection of antibody, 0.25 μg/μl of MCF-7 nuclear extract was injected for 5 min, followed by inflow of running buffer for 15 min as an actual dissociation phase (Figure 5a). Based on the values from the first 5 min of dissociation phase (Figure 5b), a bi-exponential equation was established using

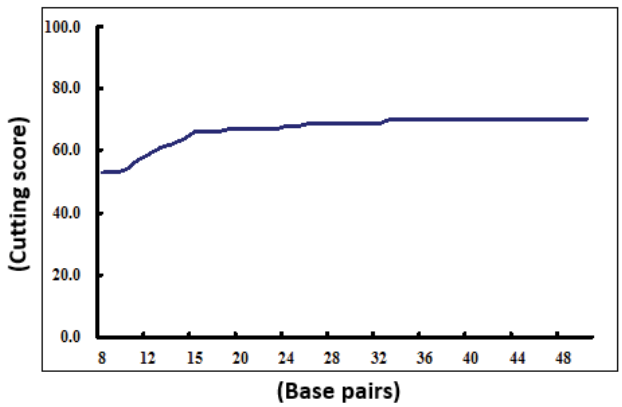

Figure 2: The cutting scores rise with increase of DNA base pairs in length.

W3DIMSUM 1.5Beta:

$$Y = Y0 + A1 \times e^{(-T/Kd)} + A2 \times e^{(-T/Kd)}$$

where Y is the value at the time of T, Y0 is the offset value for Y, A1 and A2 are the initial values, and Kd is the dissociation constant for ERα complex from crude nuclear extract. The values were extrapolated and plotted for 10 min (Figure 5c). A nearly perfect overlapping was shown when merging the dissociation phase (Figure 5d) in running buffer (Figure 4a) and extrapolation (Figure 5c). The results indicate the feasibility to further analyze the dissociation phase.

After subtraction of values from NoMf-32 (Figures 3b and 3d) from EREw2 or EREm32, the dissociation phase for ERα or ERα complex from crude nuclear extract was fitted mono-exponentially (Table 2):

$$Y = Y0 + A1 \times e^{(-T/Kd)}$$

Statistical analysis of goodness-of-fit (Table 3) showed that Degrees of Freedom from ANOVA linear fitting was 98 for EREw32 and 99 for EREm32 with bound ERα, being better fitted than that with bound ERα complex. Similar tendency could also be identified in results from Reduced Chi-squared (Chi-Sqr) and Adjusted (Adj.) R-Square test. The Residual vs. Independent plot from Residual Plot Analysis (Figure 6) showed that most of the residuals are distributed within ± 0.5 of range along the time axis. Based on this function and parameters of regression (Table 2), the values of dissociation were extrapolated after inflow of antibody and plotted for the bound ERα (Figure 3b) or ERα complex from crude nuclear extract (Figure 3d). Calculation of AUC

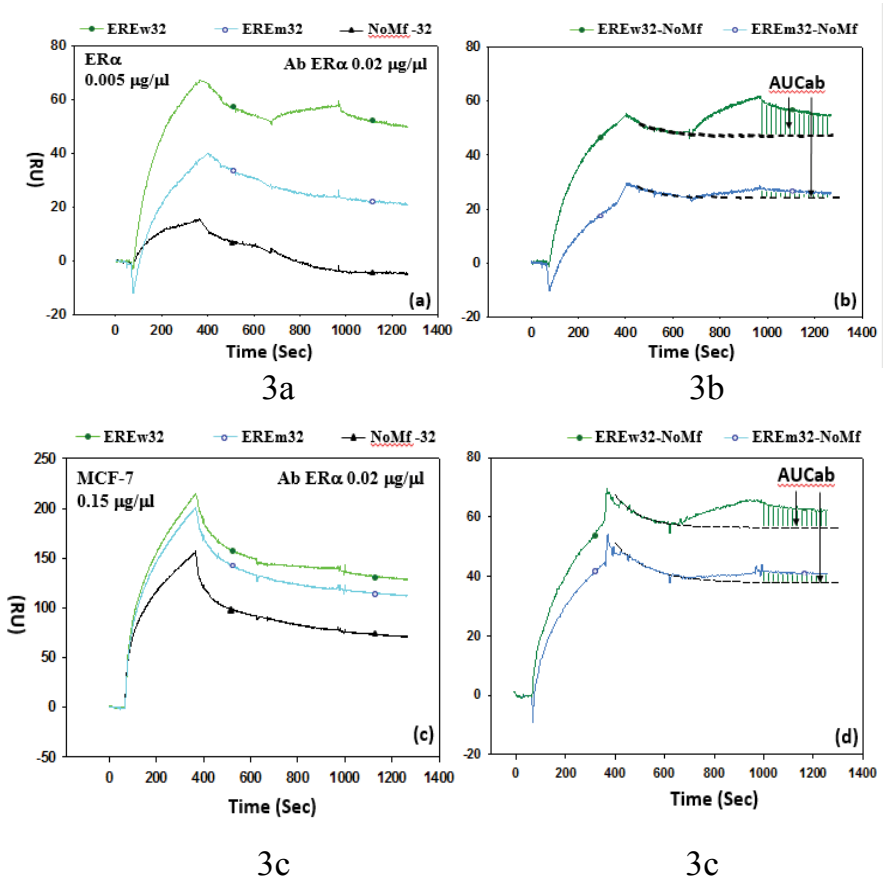

Figure 3: Sensorgrams showing binding of recombinant ERα or ERα in crude nuclear extract. After association of 0.005 µg/µl of recombinant ERα (a, b), or 0.15 µg/µl of nuclear extract (c, d), anti-ER was then injected. Sensorgrams were plotted after subtraction of responses from vacant flow cell (a, c) or that of immobilized with NoMf-32 (b, d). The dashed lines were extrapolated and plotted based on regression of dissociation phases for bound nuclear proteins (Table 2). Area Under Curve for antibodies (AUCab) was indicated based on Table 4.

Oligo	Para	ER α	MCF-7
EREw32	Y0	53.28 ± 0.16	56.46 ± 0.58
	A1	171.07 ± 24.88	662.47 ± 98.14
	Kd	123.29 ± 6.21	97.97 ± 13.75
EREm32	Y0	24.25 ± 0.21	35.68 ± 1.08
	A1	190.56 ± 30.91	101.92 ± 20.68
	Kd	113.03 ± 9.34	202.98 ± 27.23

Where, *-parameters for exponentiation; $Y\text{-}Y0+A1\times e^{(-T/Kd)}$

Table 2: Results of fitting dissociation phases.

Statistics	Erα		MCF-7	
	EREw32	EREm32	EREw32	EREm32
Number of Points	101	102	86	96
Degrees of Freedom	98	99	96	97
Reduced Chi-Sqr	0.02	0.03	0.42	0.35
Adj. R-Square	0.99	0.97	0.95	0.94

Table 3: Goodness-of-fit for dissociation phases.

Area Under Curve	Erα*		MCF-7#	
	EREw32	EREm32	EREw32	EREm32
AUCan	12652.95	8264.85	12262.77	5354.14
AUCnp	6159.21	7581.72	10661.72	4930.61
AUCab	6493.74	683.13	1601.72	423.53

Where, *- AUCab: EREw/m32 = 9.51; #-AUCab: EREw/m32 = 3.78

Table 4: Amount of bound anti-ERα antibody.

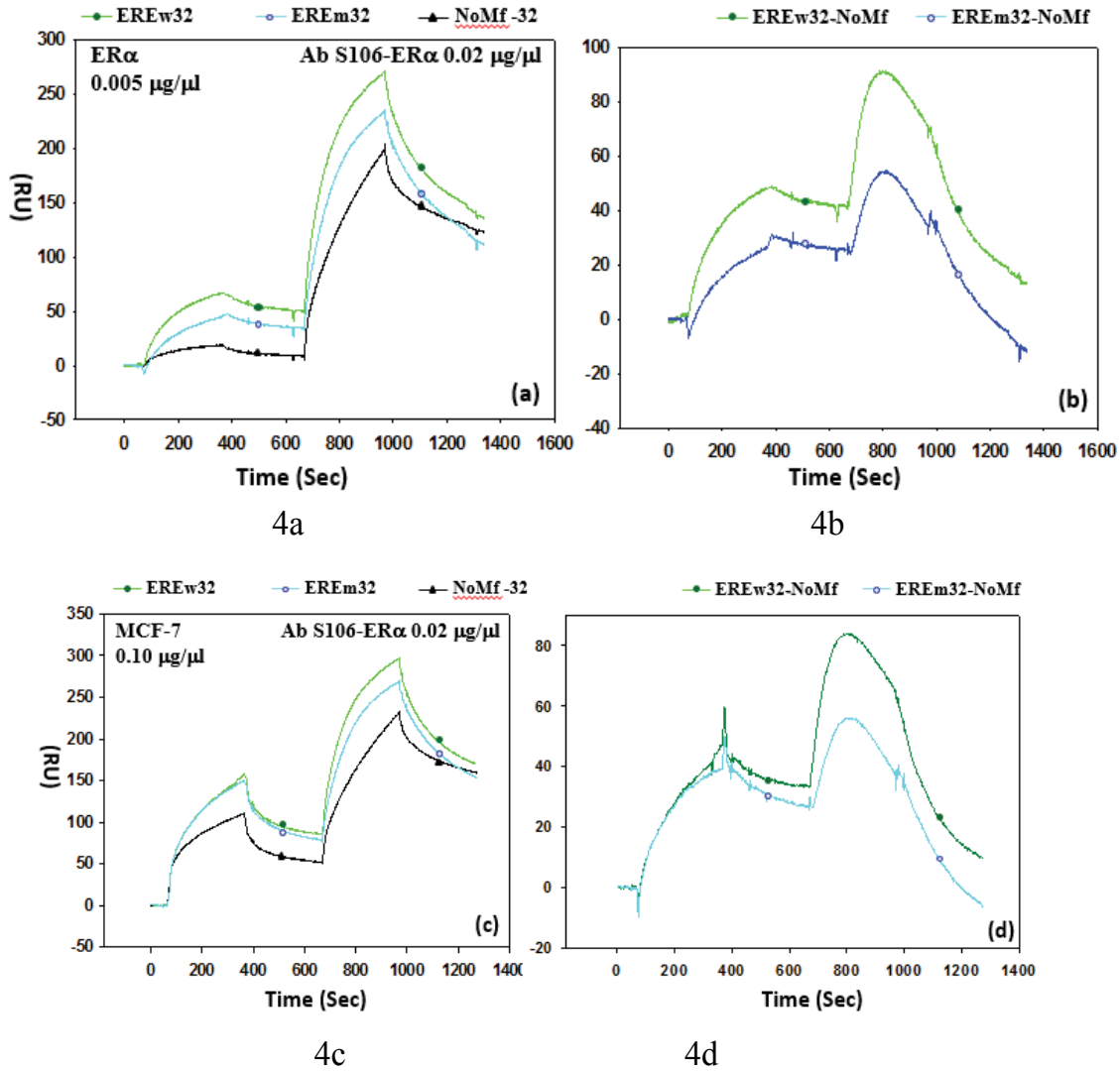

Figure 4: Binding activities of phosphorylated (S106) ERα. After association of 0.005 µg/µl of recombinant ERα (a, b), or 0.15 µg/µl of nuclear extract (c, d), anti-pS106-ER was then injected. Sensorgrams were plotted after subtraction of responses from vacant flow cell (a, c) or that of immobilized with NoMf-32 (b, d).

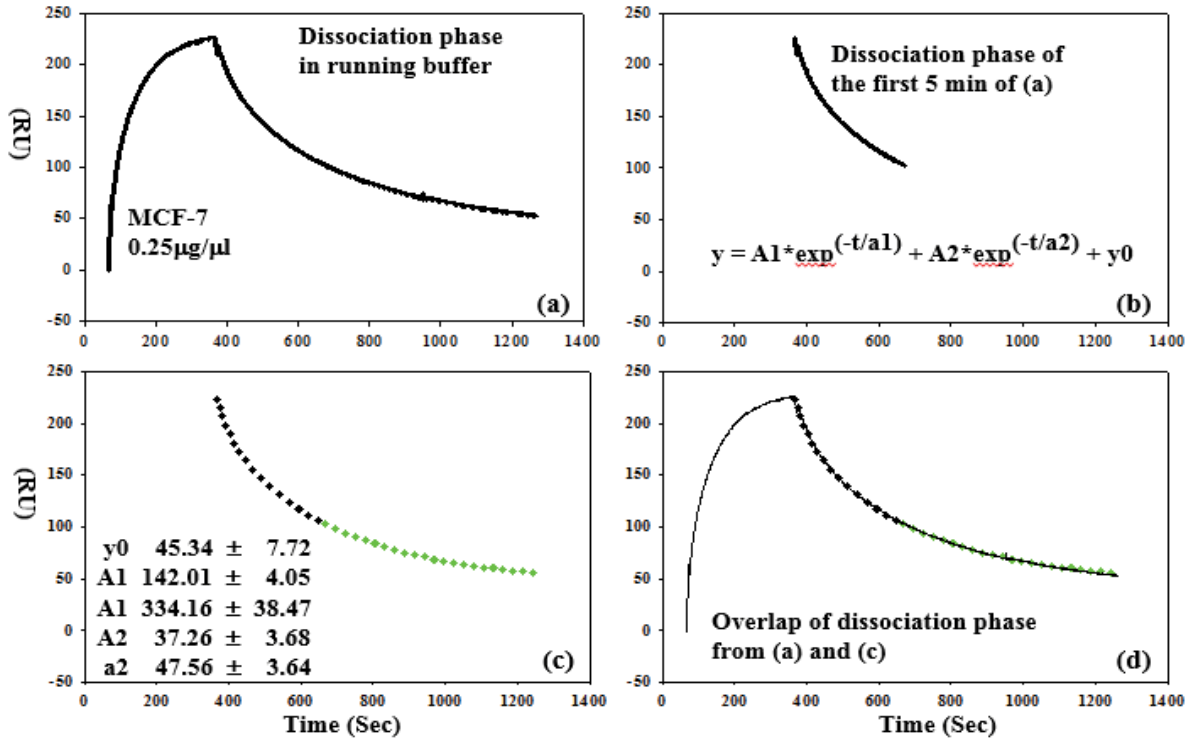

Figure 5: Fitting dissociation phase of bound ERα complex from nuclear protein on EREw32. Crude nuclear extract was injected for 5 min, followed by a dissociation phase in running buffer for 15 min (a). The first 5 min of dissociation was fitted bi-exponentially (c), and the extrapolated curve was merged with the actual dissociation phase (d).

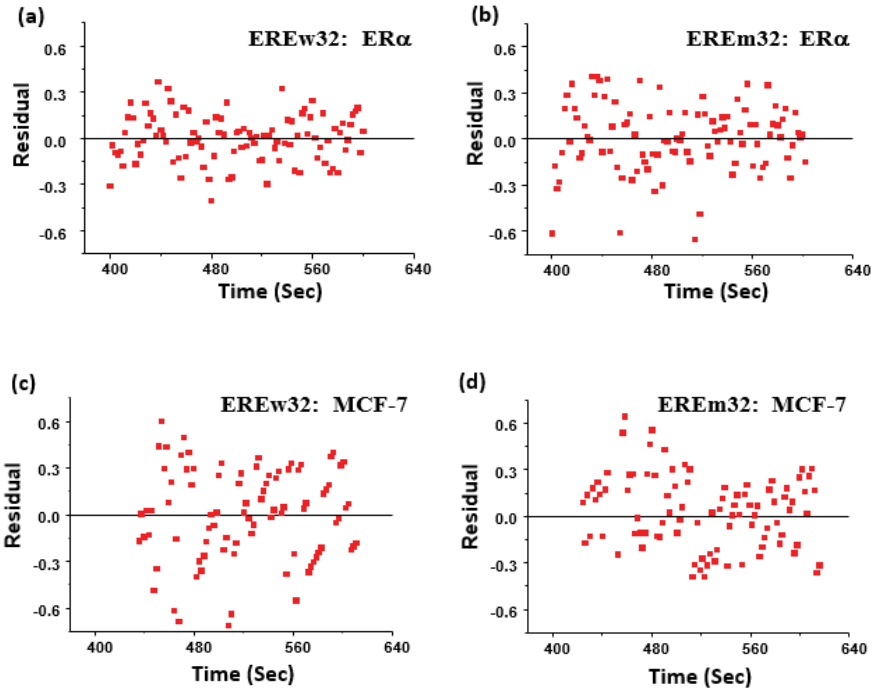

Figure 6: Residuals showing goodness-of-fit of exponential dissociation for recombinant ERα bound to EREw32 (a) or EREm32 (b), or for ERα complex in crude nuclear extract bound to EREw32 (a) or EREm32 (b).

showed that the bound antibodies (Table 4) against recombinant ERα on EREw32 was 9.51 times more than that on EREm32 (AUCab: EREm/w32). On the other hand, the bound antibodies on EREw32 against ERα from crude nuclear extract were 3.78 times more than that on EREm32 (AUCab: EREm/w32).

Discussions

Although Biacore has been extensively used in measurement of association between molecules, there are only a few reports concerning the protein-DNA complex in crude nuclear extract [10,11,15,21]. In the present study, the DNA sequences with the least protein binding activity were identified and included as control in Biacore assay. After subtraction of the non-specific bindings to these sequences, it was found that the dissociation for the bound ERα complex from crude nuclear extract could be expressed mono- or bi-exponentially, which made it possible to extrapolate the values of bound ERα complex after inflow of ERα antibodies. Then the significant association could be defined by comparing the amount of the bound antibodies between the experimental and mutant. Our procedures made Biacore a practical technique to measure protein-DNA association in crude nuclear extract.

Artificial DNA sequences with the least protein binding activity

Due to the strong electrostatic nature and hyper sensitivity of SPR, such binding controls as the vacant flow cell, fragmented genomic DNA of E. Coli, poly [d (I-C)], or poly [d (A-T)] in some studies might make it hard to obtain reproducible results for a protein-DNA complex in crude nuclear extract. A control DNA oligo with equal length to that of the experiment but specific binding motifs is essential to obtain reproducible, comparable, and reliable results. As the DNA sequences identified in the present study are 48 base pairs in length, they could cover most of the DNA sequences that specifically bind proteins. In addition, because both to recombinant ERα or ERα complex in nuclear protein, the binding value is always the lowest for our artificial DNA sequence with the least protein binding activity, such sequences could be set as a universal control for experiment *in vitro* just like in the present study.

When mutation of a DNA fragment is required for such kind of experiments as in the present study, it could be generated theoretically using such software available nowadays as TransFac public or professional, or other software alike. However, it is not known regarding a theoretical threshold value around which DNA fragment could not bind to transcription factors specifically. Both theoretically and experimentally, our results indicate the existence of artificial DNA sequences that do not bind nuclear proteins specifically. Therefore, the cutting score for TransFac in our experiments could be taken into consideration in design of a mutant oligo.

Exponential order and goodness-of-fit

It is considered [17] that the criteria for discrimination of the exponential order are still a controversial issue, and a higher score does not necessarily indicate a better regression. Between purified molecules, mono-exponential Langmuir Model [6] is usually employed for dissociation phase of a 1:1 interaction. In the present study, implication of the exponential tendency in dissociation phase was derived from linear regression after various transformations of the binding values and time, and our data could be fitted to mono- or bi-exponential function, with the average scores being higher than 95% at default setting.

Although the actual mechanism is not known, it is possible that for numerous exponential dissociations that occur simultaneously, measured data could be fitted to a sum of exponentials. Theoretically, the exponential order depends upon the difference in their initial values and dissociation constants [17]. Accordingly, if bound proteins are close in their dissociation constants and/or initial values, the data from crude nuclear extracts could still be fitted mono-exponentially. Therefore, Langmuir Model is still applicable in simultaneous events of numerous exponential dissociations.

Extrapolation of bound nuclear proteins and antibodies

It has been empirically determined that a significant binding reaction can be expected when the ratio of the amount of antibodies to antigens is more than two fold. No clear definition, however, for the significant association has been reported for a protein-DNA complex from crude nuclear extract [10,11]. Beginning from the inflow of anti-ERα antibodies in the present study, each actual data point represents both the bound nuclear proteins and antibodies, including the non-specific signals. Although mutant ERE always exhibits lower binding value than that of wide type ERE even in crude nuclear extract, using antibody is still a reliable procedure to further confirm that a target molecule exist in bound ERα complex as in Supershift Assay. Because exponential fitting of the dissociation data allows extrapolating the amount of bound nuclear proteins and corresponding antibodies in the dissociation phase, it is feasible to compare the amount of bound antibodies between the experimental and mutant statistically to define the significant association.

Conclusions

Our results showed that both theoretically and experimentally the existence of DNA fragments with the least protein binding activity, which could be used as control in SPR analysis to suppress the non-specific bindings for protein-DNA complex in crude nuclear extract. Based on the characteristics of these sequences, mutant oligo could be designed for analysis of protein-DNA binding specificity and binding site mapping. In the dissociation phase of Biacore analysis, extrapolation of the bound molecule complex from crude nuclear extract makes it possible to determine the significant association of the corresponding antibodies for a target molecule. By following these procedures, Biacore assay could be used just like a Supershift Assay on chip to measure protein-DNA association in crude nuclear extract with reproducible results.

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Conflict of Interest

The authors declare that they have no competing interests.

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