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Multi-strand Binding of Nuclear Factors to a Repressor of Mouse Mammary Tumor Virus Transcription Can Be Distinguished Kinetically

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NRE1 is a DNA sequence element in the long terminal repeat of mouse mammary tumor virus that represses viral transcription in mature T cells. In addition to double-stranded binding activity, factors in Jurkat T cell nuclear extracts bind specifically to each of the two single-strands of NRE1. Here we show that binding to the three forms of NRE1 can be distinguished kinetically. The on rates for double, upper and lower-strand NRE1 binding were 1.5, 3, and 11 min,respectively. Binding was extremely stable with off-rates varying from 30 and 60 min for double and upper-strand binding to 12 h for lower-strand binding. In addition, a trucated form of NRE1 that is only bound as a double-strand was observed to have an on rate of binding of 4 min and an off rate of 4 h.

Mouse mammary tumor virus (MMTV), is a retrovirus that activates protooncogenes flanking viral integration sites (1, 2). MMTV directed gene expression and tumorigenesis is normally highly specific for mammary gland (3-5). Horizontal transmission of MMTV occurs through a poorly defined T cell–mediated mechanism that involves expression of a superantigen encoded in the viral LTR (6). We have previously reported that the long terminal repeat (LTR) of MMTV contains a polypurine/polypyrmidine DNA sequence element (NRE1) that acts as a repressor of viral transcription in Jurkat T cells (7). Mutant viruses, lacking a portion of the LTR that includes NRE1, have elevated rates of transcription (8-15) and are efficient promoters of T cell lymphoma (15-20).

Using nuclear extracts from Jurkat cells, we have reported that NRE1 is specifically bound by at least three factors with sizes, estimated from DNA crosslinking, of 50, 80 and 95 kDa (7). Unusually, specific binding was observed to each of the separated single strands in

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addition to double stranded NRE1. U.V. crosslinking assays showed that the 80 kDa factor could be crosslinked to double–stranded NRE1 and to the purine–rich strand alone. The 95 kDa factor could only be crosslinked to the purine–rich strand while the 50 kDa factor was only detected on the pyrimidine–rich strand.

In order to further characterize the binding of these factors to NRE1 we have analyzed the kinetics of binding of nuclear factors to the three forms of NRE1, as well as to a double stranded truncated form of NRE1 which does not support single strand binding. Our results show that the binding activities can be distinguished kinetically and that binding to all forms of NRE1 is remarkably stable.

MATERIALS AND METHODS

Oligonucleotides: Oligonucleotides were synthesized on a Beckman Oligo 1000 synthesizer and as required, purified through a 20% polyacrylamide DNA sequencing gel. MTV upper–strand = 5'-AACTGAGAAAGAGAAAGACGACA-3', MT upper–strand = 5'-AACTGAGAA AGACGACA-3', MTV lower–strand = 5-TGTCGTCTTTCTCTTTCTCAGTT-3'.
Cells, Nuclear Extracts and Electrophoretic Mobility Shift Analysis (EMSA): Jurkat cells were cultured in RPMI with 10% fetal calf serum as previously described (7). Crude salt extracts of nuclear binding proteins were prepared according to Dignam (21) , as previously modified (7). For EMSA, incubations were performed in binding buffer (12 mM HEPES pH 7.9, 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 200–2000 μg/ml BSA and 1 μg of double or single–stranded, highly sheared calf thymus DNA). In brief, to measure on rates for NRE1 binding 0.5 ng ^{32}P kinased double or single–stranded oligonucleotides were incubated with 1μg nuclear extract in a 20 μl volume for between 1 min and 4 h at 20°C. To measure off rates, binding was allowed to equilibrate for 30 min at 20°C. Subsequently, a 100 to 1000 fold molar excess of unlabeled oligonucleotides was added to the incubations and allowed to compete binding for times ranging up to 16 h. For both on and off rate measurements, all incubations were set up to end at the same time. To resolve binding, samples were loaded onto 0.8 mm thick, 4%, polyacrylamide gels (acrylamide:bisacrylamide = 40:1) in 0.5 x TBE, electrophoresed for 225 V*h, dried and exposed to NEF496 film (Dupont). Densitometric analysis of exposed films, performed on an LKB Ultroscan XL (Pharmacia) was used to calculate the $t_{1/2}$'s for binding. All experiments were carried out at least three times.

RESULTS AND DISCUSSION

To compare on rates for NRE1 binding, ^{32}P–labeled NRE1 containing oligonucleotides were incubated with Jurkat nuclear extract for between 1 minute and 4 hours. Oligonucleotide MTV contains LTR sequences between –398 and –375 centered over NRE1, whereas MT has a 6 nucleotide deletion within NRE1 that results in the loss of one copy of the GAGAAAGA overlapping direct repeat of NRE1.

The on rates for binding to the three forms of NRE1 by factors in Jurkat nuclear extract were clearly distinguishable (Figure 1). Binding to upper–strand NRE1 occurred most rapidly, with a half time of 1–2 minutes. Lower–strand NRE1 binding was slightly slower, with an on rate half time of approximately 3 minutes. By contrast, the $t_{1/2}$ of binding to double–stranded NRE1 was substantially longer at 11 minutes. Notably, the on rate for binding to double–

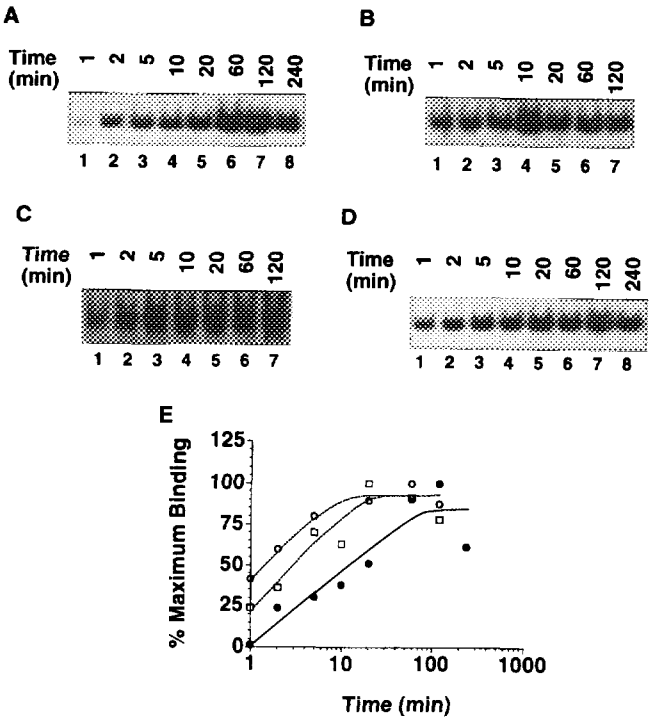

Figure 1. On rates for the binding of factors in Jurkat nuclear extracts to different forms of NRE1 determined by EMSA. EMSA were performed with Jurkat crude nuclear extract, incubated with ^{32}P–labeled double strand (*ds*) MTV (*panel A*), upper strand (*up*) MTV (*panel B*), lower strand (*lO*) MTV (*panel C*) and double strand (*ds*) MT (*panel D*) for times increasing from 1 to 240 min (lanes 1 – 8). All incubations were timed to end concurrently and maximum binding represented a shift of less than 10% of input DNA in all cases. Only the shifted complexes are shown. *Panel E*, Binding was quantified by densitometric analysis of the EMSA autoradiographs. The amount of binding for each incubation was expressed as a percentage of the maximum binding and plotted against incubation time. For clarity only three curves are shown. Time is plotted on a log scale to more clearly display curves. dsMTV – J; upMTV – E; loMTV – G.

stranded MT, at 4 minutes, was 3 times shorter than binding to the full length element, indicating that double–stranded NRE1 binding occured more efficiently to the truncated element. This suggests that removal of one copy of the overlapping NRE1 direct repeat leads to a more rapid formation of a stable protein-DNA complex on double-stranded DNA.

In competition experiments to measure off rates of the bound factors from NRE1, binding to all three forms of NRE1 was found to be extremely stable, even when competed with a 500 fold excess of specific competitor DNA's. This was somewhat surprising, as we had previously shown that a 200 fold excess of specific competitor DNA was sufficient to prevent all three binding activities (7). Upper–strand NRE1 binding activity in Jurkat nuclear extract released from the DNA with a half–time of 30 minutes (Figure 2), slow by transcription factor standards (22). Release from double–stranded full–length NRE1 was even slower, with a half–time of one hour, while the $t_{1/2}$ for release from the MT extended to 4 h.

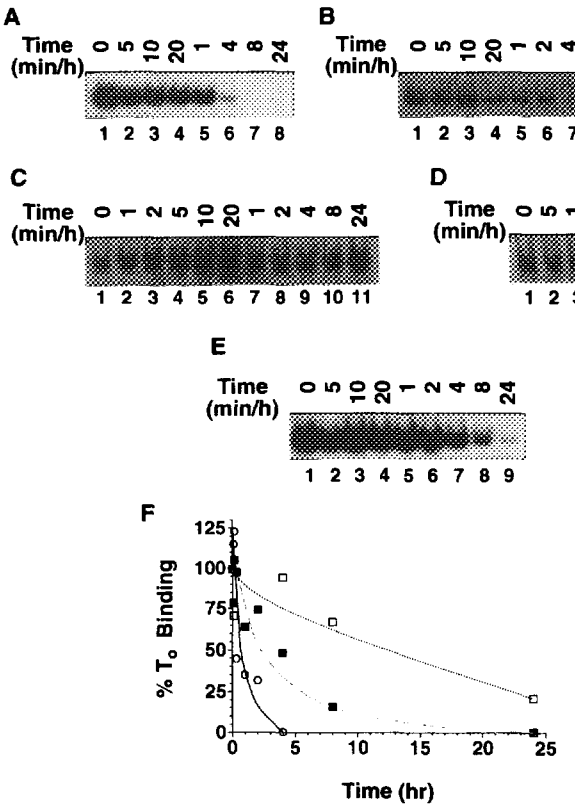

Figure 2. Off rates of NRE1 binding proteins from DNA in EMSA competition experiments. EMSA was performed with Jurkat crude nuclear extract, incubated with ^{32}P–labeled double strand (*ds*) MTV (*panel A*), upper strand (*up*) MTV (*panel B*), lower strand (*lo*) MTV (*panels C and D*) and double strand (*ds*) MT (*panel E*) and allowed to equilibrate for 30 min. Subsequently either 200 ng (*panels A, B, C, E*) or 1 mg (*panel D*) of the same unlabeled oligonucleotide was added and the incubation continued for times ranging from 0 min to 24 h (lanes 1 – 11). All incubations were timed to end concurrently and maximum binding represented a shift of less than 10% of input DNA in all cases. *Panel F*, Binding was quantified by densitometric analysis of the EMSA autoradiographs. The amount of binding for each incubation was expressed as a percentage of the maximum binding and plotted against incubation time. For clarity only three curves are shown. dsMT – B; upMTV – E; loMTV – G.

Our first attempt at competition of lower–strand binding activity with 200 ng of cold competitor showed no detectable release of the lower-strand binding factor although we have previously shown that this level of unlabeled specific competitor is sufficient to prevent binding to the same probe (7). Indeed, even when repeated with 1 μg of unlabeled lower–strand NRE1, competition was barely detectable with a half time of occupancy in excess of 12 h. The only other example of such stable binding activity for a sequence specific DNA binding protein is in a report by Schauer et al. that examined the kinetics of specific DNA binding by the glucocorticoid and progesterone receptors (23). For these nuclear hormone receptors, the loss of ligand from the receptor extends DNA occupancy from minutes to several hours. Interestingly, a related nuclear receptor, the estrogen receptor, has been shown to bind specifically to single–stranded DNA in addition to double–strand (24, 25).

Overall, the kinetics of binding to double–stranded NRE1 (on–rates) are comparable to the binding of other transcription factors to their recognition sequences, which generally seems to occur *in vitro* with half–times between 5 and 20 minutes. By contrast, little, if any information has been available on the kinetics of site–specific single–stranded DNA binding of transcription factors. Interestingly, single–stranded NRE1 binding, which requires the full length element, occurred extremely rapidly, with half times of 1.5 and 3 min for upper and lower–strand binding respectively.

One major concern with single–strand DNA binding activities is that, for them to be physiologically relevant, the binding site must be accessible in the genome. It will be interesting to see whether, and under what conditions strand separation around NRE1 can be detected *in vivo*. That the truncated version of NRE1, containing a single copy of the GAGAAAGA direct repeat, which no longer supports single–stranded binding, functions only poorly to repress transcription from the MMTV promoter in Jurkat T cells (7), suggests that the single–strand NRE1 binding activities will prove to be important for transcriptional repression.

Because of it's rapid binding and long half–time for dissociation our results suggest that an important function of the lower–strand NRE1 binding activity may be, as proposed for other single–stranded DNA binding proteins (26-30), to promote stabilization of NRE1 in a single–stranded configuration that in this instance may be critical for the effects of NRE1 on transcription. In the nucleus, exposure of single–stranded DNA binding sites depends on unwinding of DNA or the formation of alternative DNA structures such as cruciforms, parallel strands and H–form DNA, all of which are likely to be transient in nature. Rapid, stable factor binding would favor recognition and stabilization of such transient structures. This feature appears to be particularly true of single–stranded binding factors implicated in DNA replication (31-40).

The list of proven and proposed transcription factors with single–stranded DNA binding characteristics is growing rapidly. The single–stranded binding abilities of some like estrogen receptor, MyoD and Pur have been known are well established (24, 25, 31, 41). More recently, a number of additional single–stranded DNA binding activities postulated to regulate transcription have been identified (27-30, 42-50). Some of these factors also bind double–stranded DNA (24, 25, 41, 44, 47-49). Uniquely, NRE1 appears to be the first element in which both single–strands, in addition to double–strand are specifically recognized and therefore appears to be the first example of a new class of transcriptional regulatory element.

In summary, we have shown that the double and single–stranded NRE1 binding activities can be distinguished biochemically. In order to understand better the contribution of individual factors in DNA binding and to begin to reconstruct the mechanism of transcriptional repression directed through NRE1, it will be necessary to separate and identify the individual NRE1 binding components.

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