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THE INTERACTION OF A c-JUN/FOS RELATED PROTEIN FACTOR WITH THE U3 SEQUENCES OF THE MOUSE MAMMARY TUMOR VIRUS LTR

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Using polyacrylamide gel mobility shift assay we have detected a nuclear factor (NF-S) in a mouse mammary tumor cell line (GR) that binds to an upstream sequence domain (-766 to -737) near the 5'-end of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). Antibodies to the products of the Jun and Fos oncogenes interfered with the binding potential of this factor, indicating that the factor shares antigenic determinants with c-Jun/AP-1. *In vitro* translated c-Jun and c-Fos were also found to bind to the NF-S binding domain consisting of the sequence TGA(A/G)TCA that are known to be recognized by c-Jun/AP-1. Our results raise the possibility that the MMTV-LTR sequence element -766 to -737 by interacting with a Jun/Fos related protein play a role in MMTV transcription and/or the activation of *int* protooncogenes that are associated with murine mammary tumorigenesis.

Transcriptional regulation of eukaryotic promoters is modulated by a class of transcriptional control signals called enhancers (1). These sequences exhibit tissue specificity and have been identified in a number of viral genomes and in cellular genes (1,2,3). Several studies have demonstrated that the initiation of transcription results from both sequence-specific DNA-protein contacts and protein-protein interactions that occur in distinct regulatory regions of the gene. Sequence-specific motifs in which nuclear protein binds to the LTR of a number of retroviruses have been identified (4-8). In the case of MMTV, two classes of proteins, hormone receptors (HR) and nuclear factor 1 (NF-1), have been found to bind to the 3' region of the U3 LTR (9-13). It is now established that MMTV exhibits tissue tropism in its expression for the mammary glands of mice and that transcriptional activation of *int* protooncogenes caused by proviral insertion plays the major role in the development of most mammary tumors (14). Furthermore, the transcriptional orientation of the MMTV provirus in many tumors have been found to point away from the *int* genes indicating that enhancer sequences, yet to be identified, may be responsible for the expression of the *int* protooncogenes (15). Thus, it is possible that the MMTV-LTR, in addition to HR and NF-1, contain other sequence motifs that by interacting with cell

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type specific protein factor(s) may contribute to the overall functioning of MMTV in mammary tumorigenesis. Our results show that mammary tumor cells, but not normal mammary cells, express c-Jun/Fos related protein that binds to the MMTV-LTR.

MATERIALS AND METHODS

Nuclear extracts and gel mobility shift assay: Mouse mammary tumor cells, GR, and normal mouse mammary cells from BALB/c and C57BL mice were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics in a atmosphere containing 5% CO_2. Nuclear extracts were prepared according to Dignam *et al.* (16) and contained 2-8 mg protein per ml. 20 µg nuclear extract was used in each assay in a final volume of 30 µl containing 0.5-1.0 ng ^{32}P-labelled oligonucleotide, 500 ng poly(dI-dC) as nonspecific competitor, 50 mM Tris-HCl, pH 7.9, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 30 µg/ml Bovine Serum Albumin (BSA), 1 mM DTT and 4% Ficoll. After 30 min at 4°C, the protein-DNA complex was resolved on 5% non-denaturing polyacrylamide gel containing 1X TAE (Tris-acetate, EDTA). Binding with c-Jun and c-Fos was performed with the following modifications. *In vitro* translated c-Jun and c-Fos were incubated for 20 min in 20 µl reaction buffer containing 20 mM HEPES, pH 7.0, 50 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 500 ng poly(dI-dC), 0.2 mM EDTA, 0.5 mM DTT, 4% Ficoll and ^{32}P-labelled oligonucleotide. For Jun/Fos complex formation, the mixture was incubated for 60 min on ice, before ^{32}P-labelled oligonucleotide was added. The reaction mixtures were separated on 4% polyacrylamide gel containing 0.5X TBE (Tris-borate EDTA).

DNA fragments and oligonucleotide labelling: Plasmid DNAs were digested with appropriate restriction enzymes and isolated form agarose gels using NA45 DEAE membranes (Schleider and Schuell). The oligonucleotide 5'-ACTATGTTAAGAAATGAATCATTATCTTTT-3 and its complementary strand were synthesized and purified with HPLC. Both strands were annealed and used for binding assays. DNA fragments and double-stranded oligonucleotides were labelled with γ-[^{32}P]ATP using 5'-end labelling system from IBI.

Reagents: Restriction enzymes were purchased either form Promega or IBI and used according to manufacturer's description. *In vitro* translated c-Jun and Fos proteins and Fos antibody were gifts from Inder Verma (Salk Institute, San Diego). c-Jun antibody was a gift from Robert Tjian (University of California, Berkeley).

RESULTS AND DISCUSSION

First we determined whether or not there is a specific nuclear protein(s) in MMTV producing mammary cells that will interact with a U3 segment of the MMTV-LTR. As control we used a 498 bp *Bgl*II-*Rsa*I fragment (-1232 to -734), isolated from the plasmid pGR102 (17), in the binding assay with a nuclear extract. An example of the pattern of binding of a nuclear protein(s) derived from GR cells with the 498 bp DNA fragment is shown in Fig. 1A. Studies with a number of different DNA samples as competitors in the binding assay led us to conclude that the binding thus observed was specific to the MMTV-LTR. Having established the specificity of binding attempt was made to construct a preliminary map of the binding domain. For this purpose, overlapping DNA fragments encompassing the *Bgl*II (-1232) site and the third *Rsa*I (-362) site (160 bp upstream to the HR binding domain, GRE) were isolated and used in the gel retardation assay. The results of this study is

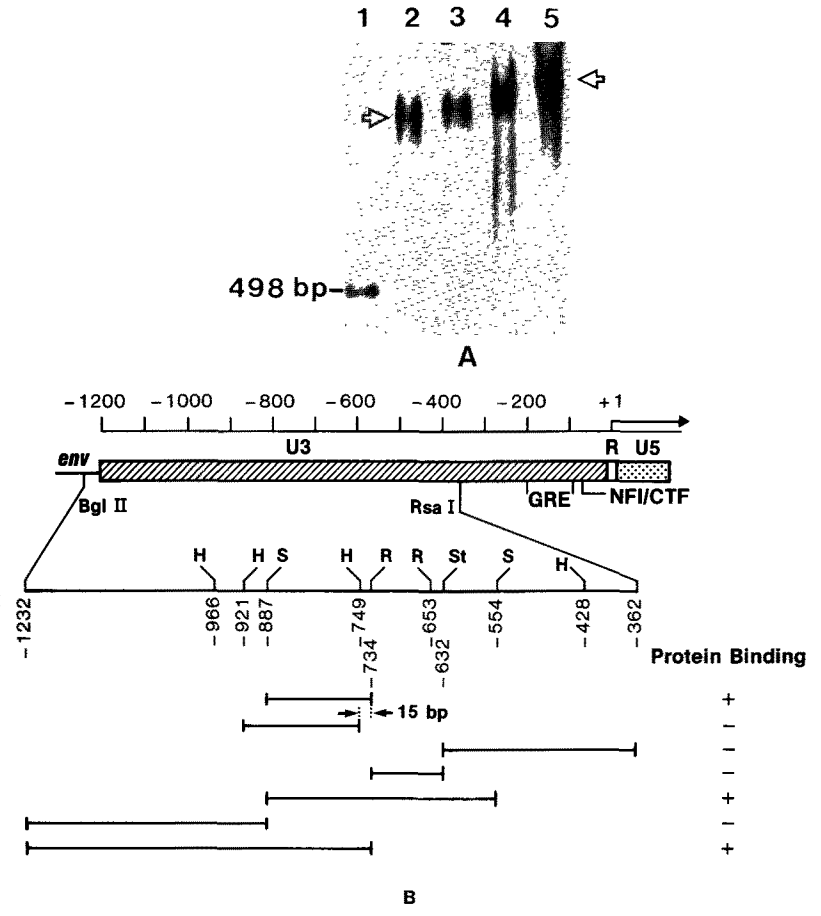

Fig. 1. Demonstration of the presence of a DNA binding factor(s) in a mouse mammary tumor cell line (GR) that forms complexes with the MMTV-LTR. Panel A shows an example of the interaction between different amounts of the protein factor and a 498 bp *Bgl*II-*Rsa*I (-1232 to -734) fragment of the LTR. The amounts of nuclear proteins used in the assays were 0 (lane 1), 1 (lane 2), 2 (lane 3), 3 (lane 4) and 5 μg (lane 5). Panel B summarizes the results of the experiments that identified the binding domain of the nuclear factor. Overlapping DNA fragments generated by enzymatic digestion of the LTR were isolated and tested in the binding assay. The notations "+" and "-" represent the presence and absence of the protein binding, respectively. As can be seen the *Sau*96I-*Rsa*I fragments (-887 to -734) gave positive result, while the *Hinf*I-*Hinf*I (-921 to -749) was negative, indicating that the binding occurred at around the *Hinf*I site (-749), which is 15 bp upstream to the -734 *Rsa*I site. H, *Hinf*I; R, *Rsa*I; S, *Sau*96I; St, *Stu*I.

summarized in Fig. 1B. Two DNA fragments defined the major binding domain: a protein-DNA complex was found to be formed with a 153 bp *Sau*96I-*Rsa*I (-887 to -734) fragment but not with a 174 bp *Hinf*I-*Hinf*I (-921 to -749) fragment indicating that the binding occurred within the 15 bp *Hinf*I-*Rsa*I domain.

Because of the difficulty in isolating by enzymatic digestion small DNA fragments from the plasmid containing the binding domain and of the need for the generation of mutation in the binding domain we tested the binding ability of a 30 bp synthetic oligodeoxynucleotide (18), 5'-ACTATGTTAAGAAATGAATCATTATCTT-

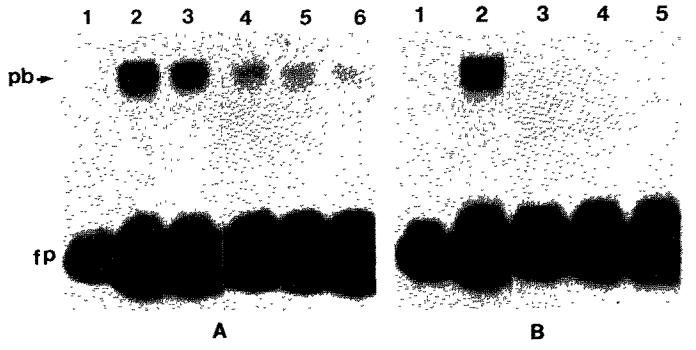

Fig. 2. Gel retardation analyses using a 30 bp synthetic oligonucleotide. Nuclear extracts (20 µg) were prepared from mammary tumor cells (panel A, lanes 2-6; panel B, lane 2) and normal mammary epithelial cells (panel B, lanes 3-5) incubated with ^{32}P-labelled, double stranded, blunt ended, oligonucleotide containing the MMTV-LTR sequences 5'-ACTATGTTAAGAAA-TGAATCATTATCTTTT-3', in the presence of 1.0, 2.5, 5.0 and 10 ng (panel A, lanes 3-6, respectively) and in the absence of (panel A, lane 2) Sau96I-RsaI (-877 to -734) competitor DNA fragment that contains the NF-S binding site. Lane 1 in both panels represents the probe alone. Binding reactions were performed as described in the legend of Fig. 1. Protein-DNA binding (pb) and free DNA probe (fp) are marked.

TT-3' (-776 to -737), that included the 15 bp putative nuclear protein binding domain. As shown in fig. 2A, apparently the same NF-S protein was binding to the synthetic DNA since competition experiments using unlabelled *Sau*96I-*Rsa*I and *Bgl*II-*Sau*96I fragments showed that, unlike the *Bgl*II-*Sau*96I fragment (data not shown), increasing amounts of the *Sau*96I-*Rsa*I fragment decreased the intensity of the complex in a quantitative manner (lanes 3-6).

In order to determine if the binding factor is also expressed in normal mammary cells we tested the nuclear extracts of two normal mouse mammary epithelial cells that we established from the mammary glands of young BALB/c and C57BL mice (Sarkar, unpublished results) and of a clonal derivative of another normal mammary gland cell line of BALB/c mouse origin developed by others (19). As shown in Fig. 2B, unlike the nuclear extracts from mammary tumor cells, the extracts from the normal mammary cells did not show any binding activity with the 30 bp synthetic probe. These observations thus defined a sequence element in the MMTV-LTR that may have an *in vivo* function in the regulation of MMTV expression. As can be seen the 30 bp nucleotide sequence contains a sequence motif of TGAATCA that is homologous to the tumor promoting agent, 12-0-tetradecanoyl-phorbol-13-acetate, (TPA) response element (TRE) that has been found to be present in a number of viral and cellular genes (see ref. 20 and table 1). Such a sequence element is also recognized by a protein product of the oncogene c-Jun as well as by the transcription factor AP-1 (20-24). Thus, we suspected the possibility of c-Jun/AP-1 binding to the 30 bp U3-MMTV LTR.

Recent studies have shown that Jun forms homodimer in the absence of Fos and heterodimer with Fos even in presence of Jun and that the Jun-Fos heterodimer bind to DNA with a higher affinity than Jun homodimers (24). Furthermore, Fos and Jun

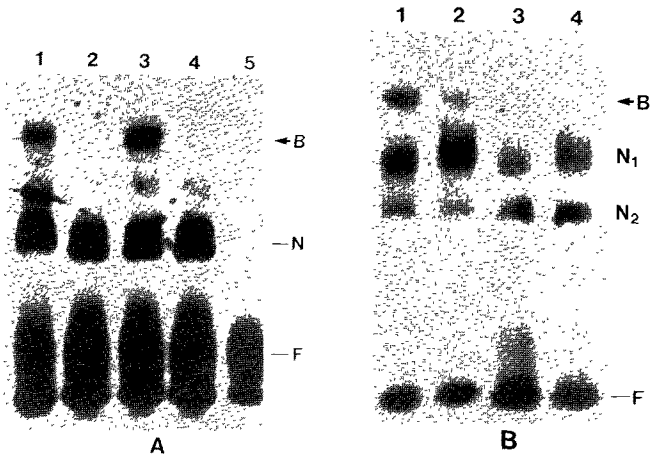

Fig. 3. The demonstration of the binding of c-Jun and c-Fos and the effect of anti Jun and Fos antibodies on the binding of NF-S to the MMTV-LTR. Panel A: *in vitro* translated c-Jun and c-Fos were used in the binding assay. Note that 1 µl (lane 2) or 2 µl (lane 4) of c-Fos did not bind to the MMTV-LTR, while 1 µl of c-Jun did bind (lane 1). The c-Jun/Fos protein complex was found to bind to the LTR sequences with higher affinity (lane 3) than c-Jun alone (lane 1). Panel B: Jun and Fos specific antibodies (2 µl) were mixed with 20 µg nuclear extracts and incubated overnight at 4°C. As control, nuclear extracts were treated with an equal volume of normal serum (lane 1). Note that Jun antibody disrupted the formation of protein-DNA complex partially (lane 2), while Fos antibody alone (lane 3) or in combination with anti-Jun antibody (1:1) prevented the complex formation completely (lane 4). B, N1 and N2 represent specific and non-specific binding, respectively; F, free oligonucleotides.

have been shown to form heterodimeric complexes through a leucine zipper in nucleoprotein complex formation with greater affinity than Jun alone (25-27). In order to test whether or not Jun/Fos proteins bind to the MMTV-LTR, we performed gel retardation assays using *in vitro* translated Jun and Fos proteins. As can be seen in Fig. 3A, lane 3, Jun and Fos together, formed a nucleoprotein complex with the 30 bp MMTV-LTR sequence with a higher affinity than Jun alone (lane 1), whereas Fos did not form such a complex (lane 2), even with an increased amount of the protein (lane 4). The extent of NF-S binding was found to be similar to the Jun-Fos complex.

Additional experiments to demonstrate the relatedness between Jun/Fos and NF-S proteins included the studies of the efficiency with which antibodies against Fos and Jun inhibited the binding of the NF-S to MMTV-LTR sequence. As shown in Fig. 3B Fos antibody alone (lane 3) and with Jun antibody (lane 4) inhibited the formation of nucleoprotein complex completely whereas Jun antibody partially disrupted the complex (lane 2). These results demonstrate that NF-S is related to both c-Jun/AP-1 and Fos and strongly suggest that the binding factor NF-S present in GR cells binds to the same sequence motif, e.g. TGAATCA in the MMTV-LTR to which Jun and Jun-Fos complexes bind.

In order to provide some direct evidence for the assumption that the TGAATCA sequence motif is involved in the binding of NF-S, we synthesized a 30 bp mutant oligonucleotide and tested its effectiveness as a competitor on the binding of NF-S to

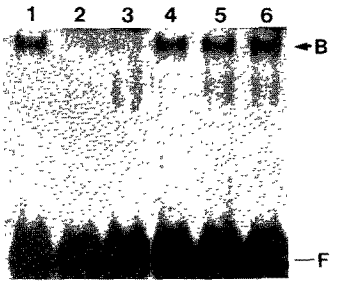

Fig. 4. The effect of point mutations of the TRE on the binding of NF-S to the MMTV-LTR. Oligonucleotide with point mutations within TRE (from G to T, and C to A; underlined) in TTAATAA was used in the competition experiment. Binding reactions were carried out (as outlined in the Materials and Methods) in the presence of 2.5 and 5 ng (lanes 2 and 3) unlabelled wild type oligonucleotide and in the presence of 2.5, 5.0 and 10 ng of the mutant oligonucleotide (lanes 6, 7, and 8), respectively, as competitor DNAs.

normal 30 bp probe. The mutant DNA was created with only two base changes in the putative binding domain: TGAATCA to TTAATAA. As expected the nonlabelled 30 bp normal oligonucleotide competed effectively with the probe in the binding of NF-S (Fig. 4, lanes 1, 2 and 3) whereas the mutant DNA did not affect the binding (lanes 4, 5 and 6). These results thus define the most probable sequences to be involved in the binding are those that include the sequence motif TGAATCA. It should be mentioned, however, that there are minor variations in nucleotide sequences of different TREs (Table 1). Thus, it appears that the heterogeneity of sequences flanking the TRE have no influence on nucleoprotein complex formation. Furthermore, it is of interest to note that, as compared to the consensus sequence motif, the fourth nucleotide of most of the TREs is G; whereas in the case of MMTV it is A. This substitution does not seem to have any effect on the binding potential of

Table 1
Nucleotide sequence comparison of TREs

TPA responsive genes	TRE	Location
hCollagenase	agcATGAGTCAgac	(-78 to -62)
rStromelysin	attATGAGTCAgtt	(-77 to -61)
hMTIIA	caaGTGACTCAgcg	(-108 to -95)
Interlukin 2	ccaTTCAGTCAgtc	(-189 to -175)
SV40	tcaATTAGTCAgca	(125 to 111)
Polyoma	tcaGTTAGTCAcac	(5125 to 5112)
MMTV-LTR	gaaATGATCAtta	(-756 to -743)
Consensus	TGA G/A TCA	

One of the two TREs from each of the SV40 and Polyoma virus is shown.

either c-Jun or Jun-Fos complex with the MMTV-LTR. Thus, as suggested by mutational analysis of TREs (20,28,29), the consensus sequence heptnucleotide TGA(G/A)TCA appears to be most favorable for binding.

While the data presented here demonstrate the presence of a protein(s), NF-S, that binds to the MMTV-LTR, the function of NF-S in MMTV biology remains to be determined. We hypothesize that 1) NF-S may affect in *trans* MMTV transcription by directly modulating the strength of the viral LTR promoter and/or by affecting the hormonal response of the promoter, and 2) the sequence to which NF-S binds is the putative enhancer element that activates the transcription of *int* oncogenes by insertional mutation. There are at least two indirect observations that led some support to this hypothesis. First, mutations outside the hormone receptor binding sites differentially affect the hormone inducibility of the MMTV promoter (30), implying that sequences other than hormone response elements are involved in the regulation of MMTV transcription, and that these sequences, as identified in the present study, are potential targets for cellular factors. Second, although the exact sequences to which NF-S and Jun proteins bind remain to be determined, our results suggest that these proteins bind to the same sequence motif TGAATCA that is present in TPA-inducible genes. In this case one would expect TPA to enhance the expression of NF-S, which in turn should increase MMTV transcription due to enhanced DNA-protein interaction. The observation that TPA indeed increases the production of MMTV particles (31) thus supports our hypothesis.

Another aspect of our studies which demonstrates that, in the context of the formation of DNA-protein complexes, NF-S shares some structural and/or functional relationships with Jun/Fos raises an interesting possibility for a role(s) of Jun/Fos in MMTV transcription and/or in mammary tumorigenesis. It is possible that in the nuclear extract of GR cells there are more than one protein factor besides Jun/Fos that form the nucleoprotein complex in the gel retardation assays. Results from other laboratories indicate that the Jun and Fos proteins appear to be associated with other nuclear proteins in transcriptional complexes (29,32,33). The possibility of NF-S being such a protein needs to be explored. It has been shown that c-Jun and the human transcription factor AP-1 are closely related to each other in both structure and function, even though AP-1 is speculated to represent different proteins with indistinguishable DNA binding properties (28). Thus we hypothesize that NF-S being a member of the Jun oncoprotein family, on its own or in complexes, may play specific roles in MMTV gene expression and/or mammary cell proliferation. Our results and the fact that the very distantly related GCN4 protein in yeast recognizes precisely the same nucleotide sequences as the human transcription factor AP-1 support this speculation (34,35). Future studies of the purification and sequences analyses of the protein factor, NF-S, as well as the evaluation of its pattern of expression in a panel of MMTV-infected normal and mammary tumor cells should establish the structure-function relationships of NF-S with c-Jun/AP-1 and its role in MMTV biology.

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