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*Articles*

# In Vitro Activity of the EWS Oncogene Transcriptional Activation Domain†

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ABSTRACT: Aberrant chromosomal fusion of the Ewings sarcoma oncogene (EWS) to several different cellular partners gives rise to the Ewing's family of oncogenic proteins [EWS fusion proteins (EFPs)] and associated tumors (EFTs). EFPs are potent transcriptional activators dependent on the N-terminal region of EWS [the EWS activation domain (EAD)], and this function is thought to be central to EFT oncogenesis and maintenance. Thus, EFPs are promising therapeutic targets, and detailed molecular studies of the EAD will be pivotal for exploring this potential. For many reasons, the molecular mechanism of EAD action is poorly understood and one major obstacle to progress is the lack of an in vitro transcription assay. Using well-characterized EAD-dependent activators and soluble nuclear extracts, we have attempted to recapitulate EAD transcriptional actvity in vitro. We report that while the EAD activates transcription strongly in vitro, the effect of EAD mutations is strikingly different from that observed in vivo. Our results therefore suggest that crude soluble extracts do not support bona fide EAD activity in vitro, and we discuss our findings in relation to future assay development and potential mechanisms of EAD action.

Pathological genomic fusion of members of the TET family [TAF15, EWS, and TLS (*1*)] to several different cellular partners gives rise to the Ewing's family of oncogenic proteins [EWS[1] fusion proteins (EFPs)] and associated tumors (EFTs) (*2*−*4*). EFPs include EWS/Fli1 (EFT, Ewings sarcoma), EWS/WT1 (desmoplastic small round cell tumor), EWS/ATF1 (clear cell sarcoma), EWS/TEC (chondrosarcoma), EWS/ZSG (small round cell tumor), TLS/ERG (myeloid leukemia), and TLS/CHOP (liposarcoma). EFPs are potent gene-specific activators with the N-terminal ∼250 residues of EWS providing a transcriptional activation domain [EWS activation domain (EAD)], and the fusion partner conferring DNA binding/promoter specificity (*2*). Aberrant transcriptional activation by EFPs is most likely central to EFT oncogenesis, but other effects of EFPs, including gene-specific transcriptional repression (*4*) or perturbation of pre-mRNA splicing (*4*), may also be important.

In addition to being oncoproteins, EFPs may also be promising therapeutic targets (*5*). EFPs are absolutely tumor-specific, their function is quite distinct from that of the parental proteins (*4*), and at least some EFPs [EWS/FLI1 (*6*) and EWS/ATF1 (*7*)] appear to play a role in tumor cell survival. Thus, detailed molecular studies of EFPs/EAD will be pivotal for exploration of novel therapeutic strategies targeting EFPs.

For many reasons, the molecular mechanism of EAD-

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[1] Abbreviations: EWS, Ewings sarcoma oncogene; EFPs, EWS fusion proteins; EFT, Ewings family tumor; EAD, EWS activation domain; IDP, intrinsically disordered protein; Gst, glutathione *S*-transferase; bZIP, basic leucine zipper; TAD, transcriptional activation domain; PIC, preinitiation complex; HAT, histone acetyl transferase; CBP, CREB-binding protein; EBV, Epstein−Barr virus.

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mediated transcriptional activation is not well-characterized. First, studies of native TET family members have not been informative for the EAD because, in contrast to EFPs, TETs only weakly activate transcription (*8*). Second, the EAD has a repetitive and highly biased composition (enriched in Tyr, Gln, Ser/Thr, Ala, Gly, and Pro), and CD spectrometry (*9*) and computational (*10*) and functional analysis (*10*) all demonstrate that the EAD is an intrinsically disordered protein (IDP) region (*11*). Thus, the EAD is not amenable to classical structural analysis. Third, similar to other long and malleable IDPs, the EAD most likely interacts with a complex array of proteins (*12*) at the hub of a protein interaction network (*12, 13*) or as a scaffold protein (*14*). Given this complexity, identification of functionally critical EAD-interacting proteins represents a significant challenge.

Functional studies of the EAD have largely been limited to transfection-based assays involving introduction of exogenous EFPs into mammalian cells. EWS/ATF1 (*15, 16*) or derivatives thereof (*10, 17*) are particularly potent transcriptional activators and have provided a useful model system. The intact EAD spans ~250 residues and is required for full activity (*18, 19*), but consistent with the presence of repetitive elements, several small EAD subregions (of ~40 residues) can functionally cooperate in cis or trans (*17*). Thus, both the composition and function of the EAD indicate a high degree of flexibility. A systematic mutagenesis of the intact EAD has recently uncovered specific EAD features critical for the transciptional and transforming activity of EWS/ATF1 and EWS/FLI1, respectively (*10*). First, multiple Tyr residues play a particularly crucial role, although an aromatic side chain is sufficient (Phe can substitute for Tyr). Thus, while the EAD is phosphorylated on multiple tyrosines (*20, 21*), major EFP functions can occur when the EAD is devoid of tyrosine phosphorylation (*10*). Second and quite remarkably, overall amino acid composition rather than specific peptide sequences confer EAD activity (*10*). From the findings described above, the EAD is proposed to be highly extended, flexible, and malleable, with several critical tyrosine residues (*10, 22*).

Detailed molecular analysis of the EAD has been greatly hindered by lack of a faithful, EAD-dependent, in vitro transcription system. One study reported that recombinant EWS/FLI1 produced in bacteria can specifically activate transcription in vitro (*9*), but it was not established whether activation by EWS/FLI1 is EAD-dependent. Using an established transciption system (soluble nuclear extracts) and the panel of informative EAD mutants described above (*10*), we have determined the transcriptional properties of the EAD in vitro. Surprisingly, although some of the proteins tested activate transcription in a highly efficient and EAD-dependent manner, the effect of EAD mutations in vitro is not correlated with that previously established in vivo (*10*). Our results therefore suggest that crude soluble extracts do not support bona fide EAD activity in vitro, and we discuss our findings in relation to assay development and potential mechanisms of EAD action.

## EXPERIMENTAL PROCEDURES

*Plasmids.* Mammalian expression vectors pZ57 and pZΔE (*17*), pZ57A and pZ57F (*10*), and pΔ167C (*18*) are as previously described. pN3Z was derived from pΔ167C by replacing the ATF1 bZIP domain with the zta bZIP domain using an NdeI site engineered for bZIP domain swaps (*23*). pN3ZA and pN3ZF were obtained by inserting HindIII/BglII-ended synthetic DNA fragments obtained by total gene synthesis (TOP Gene Technologies, Montreal, QC) directly into pZΔE digested with HindIII/BglII. Plasmids serving as templates for in vitro transcription [pZ1E4TCAT, pZ3E4TCAT, and pZ7E4TCAT (*24*), pRSVCAT (*25*), pWE4WT (*26*), and pBR322-Bal1-E (*27*)] have been previously described.

Bacterial expression vectors for 57Z and related mutants were derived from pGEX-2T as follows. pGEXvec contains a multiple cloning site, including HindIII and XbaII sites enginerred between the thrombin cleavage site and translation stop codons in pGEX-2T. pGEX-57Z was obtained by insertion of a HindIII/XbaI fragment (including the complete coding sequence for 57Z) into HindIII/XbaI-digested pGEX-vec. pGexZΔE was obtained by digestion of pGEX57Z with HindIII/BglII, end filling, and in-frame religation. pGEXZbZ was obtained by digestion of pGex57Z with HindIII/NdeI and insertion of an oligonucleotide to create the correct reading frame. pGEXZE was obtained by a three-way ligation of HindIII/NdeI-digested pGEX57Z, a HindIII/BglII fragment from p57Z, and a synthetic oligonucleotide to create an in-frame fusion between EAD1−57 and the zta bZIP domain. pGEX57ZA and pGEX57ZF were constructed by using pZ33A and pZ33F (*17*), respectively, as PCR templates and adding the missing EAD sequence within one of the PCR primers. PCR products were digested with HindIII and BglII and inserted between the HindIII and BglII sites of pGex57Z.

*Informative EAD Mutants.* The EAD mutations exploited in this study (and described in the legends of Figures 1C and 6) have been functionally characterized in vivo in mammalian cells (*10*). Multiple Tyr to Ala changes within the EAD (in native EWS/ATF1 or in the derivative proteins, 57ZA and N3ZA, containing the zta bZIP domain) impair EAD activity in vivo (*10*). Analysis of several other EAD mutations (*10*) has established that the effect of Tyr to Ala changes reflects the critical function of multiple Tyr residues and is not due to a gross malfunction engendered by the mutational burden. Specifically, a similar degree of Ala substitution in other over-represented EAD residues (Gln and Ser/Thr) has no effect on EAD activity (*10*). Thus, the Tyr to Ala mutants (57ZA and N3ZA) are valuable tools for verifying authentic EAD activity in vitro. Additional analysis also showed that Phe and Trp effectively substitute for Tyr, while Ile and His do not (*10*). Thus, it is established that the aromatic ring (but not the hydroxyl group) of tyrosine is sufficient for EAD activity in the in vivo assays employed (*10*).

*Purification of Gst−57Z Protein and Derivatives from Bacteria.* All proteins were expressed in bacterial strain BL21(DE3) and extracted and purified on ice. Cells from 300 mL of culture were resuspended in 4 mL of LB [lysis buffer (1 M NaCl, 0.5% Triton X-100, and 1× PBS)] and lysed by sonication. The lysate was clarified by centrifugation at 14000 rpm for 15 min in an Eppendorf microfuge and the supernatant mixed with 800 μL of glutathione Sepharose 4B (GE Healthcare) for 1 h. The resin was washed with 40 mL of LB, and proteins were eluted via addition of 800 μL of elution buffer (0.5 M NaCl, 20 mM reduced glutathione, and 10% glycerol). The eluate was then concentrated using

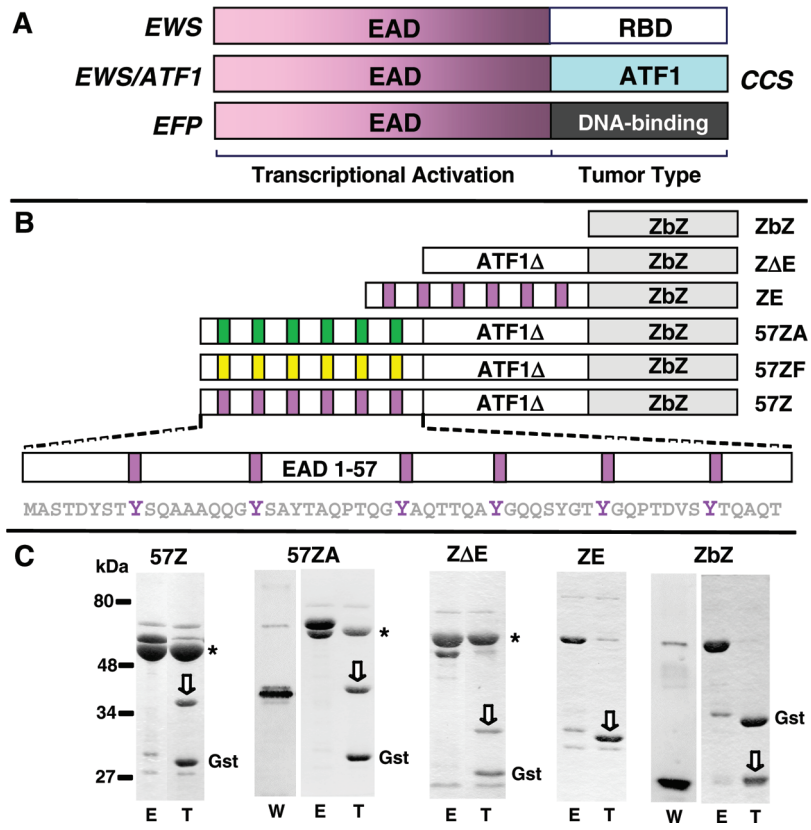


FIGURE 1: EFPs and proteins produced in bacteria. (A) EWS fusion proteins (EFPs). The normal EWS protein contains an N-terminal region [the EWS activation domain (EAD)] and a C-terminal RNA-binding domain (RBD). All oncogenic EFPs contain the EAD (at least EWS residues 1−264), lack the EWS RBD, and are differentiated by a distinct C-terminal DNA-binding domain contributed by the fusion partner (ATF1 in the case of EWS/ATF1). EWS/ATF1 is the causative agent of clear cell sarcoma (CCS) and is a potent constitutive activator of ATF-dependent promoters. (B) Structure of 57Z and mutants. 57Z contains EAD1−57, the ATF1 region present in EWS/ATF1 (ATF1Δ), and the bZIP domain of zta (ZbZ) in place of the ATF1 bZIP domain. 57Z strongly activates transcription in mammalian cells (*10*) dependent on ZbZ which mediates DNA binding. EAD1−57 contains several tyrosine residues (purple and represented by purple boxes in 57Z) that are critical for transcriptional activation in vivo (*10*). 57ZA and 57ZF are identical to 57Z except that the highlighted tyrosines are all changed to Ala (57ZA, green boxes) or Phe (57ZF, yellow boxes). (C) Purification of Gst fusion proteins. Proteins were eluted from glutathione affinity resins (E), treated with thrombin (T) to remove the Gst tag, and detected by Coomassie blue staining of SDS gels. Efficient thrombin cleavage (shown only for 57ZA and ZbZ) was monitored by Western blotting (W) using an antibody (KT3) against a C-terminal epitope tag. All protein samples (except ZbZ and ZE) contain GroEL as indicated (asterisks).

a centrifugal filter with a cutoff of 10 kDa (Ultracel YM-10 Millipore). Finally, Gst fusion proteins were cleaved at 30 °C for 2 h in elution buffer adjusted to 2.5 mM $CaCl_2$ and ~100 units/mL thrombin (GE Healthcare). The cleaved proteins were aliquoted and quick-frozen in liquid nitrogen. Purification was verified and quantified by SDS−PAGE, staining with Coomassie Blue R250, and comparison with a known BSA standard. Efficient thrombin cleavage was confirmed by Western blotting using the KT3 antibody (*28*).

Histidine-tagged G4VP16 was prepared on ice from cell lysates obtained from 300 mL of culture generally as described above for Gst fusion proteins. The lysate was mixed with 200 μL of Ni-affinity resin (NTA, Qiagen) for 1 h, and the resin was washed with 5 mL of LB, washed three times with 1 mL of 50 mM imidazole [in 10 mM Tris (pH 8.0)], and finally eluted with 200 μL of elution buffer [100 mM imidazole, 10 mM Tris (pH 8.0), and 20% glycerol]. The purified eluate containing histidine-tagged Gal4VP16 (G4VP16) was aliquoted, quick-frozen in liquid nitrogen, and stored at −80 °C.

*Preparation of Proteins from Transfected Mammalian Cells.* Each of ten 100 mm tissue culture dishes containing 50% confluent Jeg3 cells was transfected by calcium phosphate coprecipitation with 5 μg of activator expression plasmid and 15 μg of pGem3 as a carrier. Forty hours post-transfection, cells were washed with 5 mL of PBS per dish and harvested and protein extracts were prepared in either of two ways.

For 57Z and 57ZA, whole cell extracts were prepared by resuspending cells in 50 μL of buffer (per 100 mm dish) containing 10 mM Hepes (pH 8.0), 0.21 M NaCl, 0.75 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, and protease inhibitors (Complete Cocktail, Roche Diagnostics GmbH) and lysed by three freeze−thaw cycles (liquid nitrogen/37 °C). Samples were centrifuged at 12000 rpm for 1 min in an Eppendorf microfuge; the supernatant was heated at 65 °C for 5 min, and denatured proteins were removed by centrifugation at 12000 rpm for 5 min in an Eppendorf microfuge. Supernatants were pooled and then concentrated approximately 20-fold using a YM-10 centrifugal filter at 4 °C, adjusted to 20% glycerol, quick-frozen in liquid nitrogen, and stored at −80 °C.

For N3Z, N3ZA, and N3ZF, nuclear extracts were prepared from transfected cells on ice as previously described (*29*) but with some modifications. Cells were lysed in low-salt lysis buffer [300 μL per dish containing 20 mM Hepes (pH 8.0), 20 mM NaCl, 0.5% NP40, 1 mM DTT, and protease inhibitors (Roche)]. The crude nuclear pellet was

obtained by centrifugation for 20 s at top speed in an Eppendorf microfuge and resuspended in 60 $\mu$L per dish of 10 mM Hepes (pH 8.0), 0.21 M NaCl, 0.75 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, and protease inhibitors. Subsequent steps were as described above for the preparation of 57Z and 57ZA.

*In Vitro Transcription Assays.* Nuclear extract from Hela S3 cells was prepared as previously described (*30*) but with some modifications (*31*). Forty microliter transcription reactions were performed at 30 °C for 1 h in buffer containing 7.5 mM $MgCl_2$, 60 mM KCl, all four unlabeled ribonucleoside triphosphates (500 $\mu$M each), 27 $\mu$L of nuclear extract, and the indicated amounts of DNA template and test protein samples. To initiate reactions, all components except test proteins were mixed on ice, and test proteins were diluted to the desired concentration and then immediately added. Concentrations of test proteins were equalized according to DNA binding activity. For transcript detection, primer extensions were performed as previously described (*32*) using DNA primers labeled with T4 polynucleotide OptiKinase (USB) and EasyTides [$\gamma$-$^{32}$P]ATP (Perkin-Elmer), with a final ATP concentration of 3 $\mu$M and a specific activity of 2000 Ci/mmol. Primer extension products were resolved via 10% denaturing polyacrylamide−urea gels which were directly exposed to Kodak Biomax XAR film. Exposure at −80 °C typically lasted for 2 h for freshly labeled primer.

*DNA Binding Assays.* Gel mobility shift assays were performed as previously described (*17, 23, 33*). The labeled zta DNA probe contains a single zta binding site with the TTGCTAA core motif, and competitor oligonucleotides have either a wild-type zta binding site or a mutant binding site with the core motif changed to GACACAC. Purified bacterial proteins or partially purified proteins from mammalian cells were diluted as desired and then immediately added to the binding reaction mixtures. Fifteen microliter reaction mixtures, containing ~1.5 ng of labeled DNA probe and 1 $\mu$g of poly-dIdC, were incubated for 20 min at 30 °C, and half of the reaction mixture was resolved on 5% polyacrylamide gels run in 50% TBE. Gels were fixed in 50% methanol and exposed at −80 °C for ~2 h to Kodak Biomax XAR film.

*Western Blotting.* Western blotting was performed in PBS containing 3% dried milk, using monoclonal antibody KT3 (*28*) and alkaline phosphatase-conjugated anti-mouse secondary antibody (DAKO D0486). All proteins contained the KT3 epitope PPPEPET at the C-terminus adjacent to the zta bZIP domain.

## RESULTS

*Strategy for Assaying the EAD in Vitro.* Using mammalian cell (HeLa S3) nuclear extracts (*30, 31*) and a source of exogenous EAD-containing activator protein, we aimed to develop an in vitro transcription assay for the EAD. EWS/ATF1 is a particularly potent activator (*15*), and we have characterized a number of informative EAD mutants in vivo (see Experimental Procedures) in the context of EWS/ATF1 (*10*) that are critical for evaluation of authentic EAD function in vitro. However, native EWS/ATF1 could not be used to develop an in vitro transcription assay because promoters containing ATF binding sites are strongly activated by endogenous proteins (CREB/ATF1) in nuclear extracts (*32*), rendering them unresponsive to an exogenous activator.

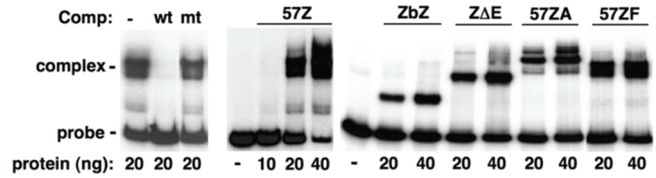


FIGURE 2: DNA binding activity of bacterially expressed proteins. Purified 57Z (20 ng) or the indicated amounts of all mutant proteins were incubated with 1 ng of labeled probe containing a single Zta binding site. DNA−protein complexes were resolved on nondenaturing polyacrylamide gels and detected by autoradiography. Positions of unbound DNA probe and DNA−protein complexes are indicated at the left. The presence of excess competitor oligonucleotide (100 ng) containing a functional (wt) or mutated (mt) zta binding site is indicated.

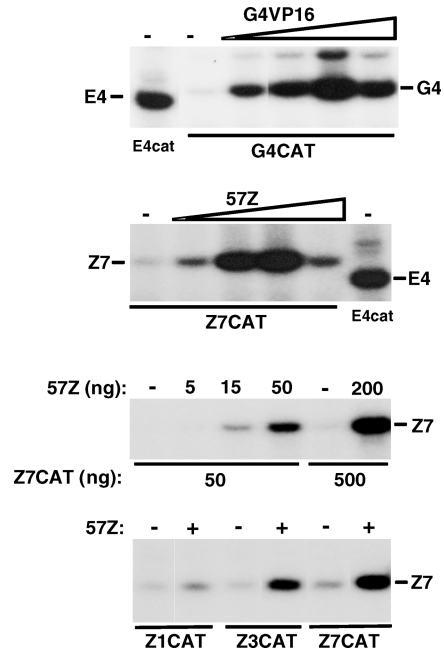


FIGURE 3: In vitro transcription using 57Z from bacteria. Transcription assays were performed in nuclear extracts using purified Gal4-VP16 (G4VP16) and 57Z proteins with their corresponding reporters G4CAT (containing five Gal4 binding sites) and Z7CAT (containing seven zta binding sites). E4cat (E4) contains the adenovirus E4 promoter which is highly active in nuclear extracts without an exogenous activator (*32*). All transcripts were detected by primer extension using a $^{32}$P-labeled CAT primer followed by autoradiography. The top panel shows the activity of G4VP16. Reaction mixtures contained 500 ng of E4cat or G4CAT and increasing amounts of G4VP16 (200, 350, 500, and 800 ng) as indicated. The second panel shows the activity of 57Z. Reaction mixtures contained 500 ng of E4cat or Z7CAT and increasing amounts of 57Z (125, 250, 500, and 1000 ng) as indicated. The third panel shows the effect of template concentration on 57Z. Transcription reaction mixtures contained different amounts of Z7CAT and 57Z as indicated. The bottom panel shows the effect of zta binding sites on 57Z activity. Transcription reaction mixtures contained 500 ng of 57Z and different reporters with one (Z1CAT), three (Z3CAT), or seven (Z7CAT) zta binding sites.

Moreover, endogenous ATF1/CREB could also impact EWS/ATF1 activity in vitro via dimerization. To circumvent these problems, we employed EWS/ATF1 derivatives previously characterized in vivo (*17*) that contain the EBV zta bZIP domain in place of ATF1. A zta-responsive reporter containing zta binding sites (Z7E4TCAT, termed Z7CAT throughout) has only low activity in HeLa cell nuclear extracts (Figure 3), thereby allowing a response to an exogenous activator.

In light of the unstructured nature of the EAD (*10*) and ATF1/zta (*23*) and the lack of requirement for EAD tyrosine phosphorylation in vivo (*10*), we initially reasoned that proteins produced in bacteria might well retain function. One additional barrier, however, was presented by the insolubility of most EAD-containing recombinant proteins in bacteria (ref *9* and our unpublished results). We found, however, that a Gst fusion protein (Gst−57Z) containing EAD1−57, ATF1, and the zta bZIP (Figure 1) was relatively soluble. Though it contains only a small region of the EAD, 57Z activates transcription to very high levels in vivo (*10, 17*) and, crucially, in a manner that mimics the intact EAD (*10*). We therefore attempted to establish the in vitro assay using HeLa cell nuclear extract, bacterially expressed 57Z and Z7CAT as the DNA template.

*Purification of 57Z and Derivatives from Bacteria.* The proteins shown in Figure 1 were expressed as Gst fusions in BL21(DE3) bacteria. 57Z contains a total of nine EAD Tyr residues which, together, are crucial for in vivo activity (*10*). 57ZF [active in vivo (*10*)] and 57ZA [inactive in vivo (*10*)] have six of the above Tyr residues changed to Phe and Ala, respectively. ZbZ contains only the zta bZIP domain; ZΔE lacks the EAD, and ZE lacks the portion of ATF1 present in EWS/ATF1. Gst fusions were purified using glutathione Sepharose, eluted with glutathione, and treated with thrombin protease to release the native proteins. Purified proteins were analyzed by Coomassie blue staining of SDS gels, and efficient thrombin cleavage was confirmed by Western blotting (Figure 1). A protein of ~60 kDa copurified with some of the proteins and this protein is almost certainly the bacterial chaperone GroEL. Although we were able remove GroEL by incubation with ATP and denatured bacterial proteins as previously described (*34*), such treatment resulted in prohibitive loss of protein via entrapment on the Gst resin. GroEL was copurified with 57Z and 57ZA and ZΔE (all of which contain ATF1) but not with ZE or ZbZ (each of which lacks ATF1), indicating that GroEL binds to ATF1 and not to the EAD or zta bZIP domain. In addition, the presence of GroEL did not affect DNA binding activity (see Figure 2), and the proteins were not aggregated (Figure 2). Together, the observations described above suggest that the presence of GroEL is unlikely to affect EAD transcriptional activity, and we therefore proceeded to functionally characterize 57Z produced in bacteria.

*DNA Binding Activity of 57Z from Bacteria.* To functionally characterize bacterially derived 57Z, we first tested DNA binding activity (Figure 2) using a gel mobility shift assay previously described for 57Z from mammalian cells (*17*). 57Z formed a specific complex with a $^{32}$P-labeled DNA probe containing a single zta binding site, with binding contested by excess oligonucleotide containing a zta binding site (wt) but not by the corresponding mutated zta site (mt). Titration experiments in the dose response range showed that the DNA binding activities of 57Z, ZbZ, ZΔE, 57ZA, and 57ZF were comparable; 20 ng (~40 nM) of 57Z was typically required to bind 50% of the DNA probe (2.5 nM), indicating a $K_d$ of ~40 nM, consistent with published results for renatured EWS/FLI1 (*9*) or native zta protein (*24*) produced in bacteria.

*Transcriptional Activity of 57Z from Bacteria.* Next we tested the transcriptional activity of bacterial 57Z in nuclear extracts. We used a promoter (Z7CAT) containing seven zta binding sites that we previously employed to characterize

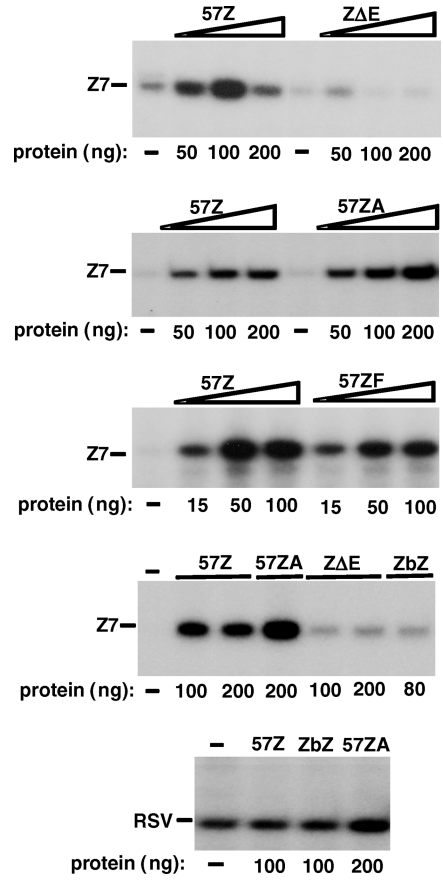


FIGURE 4: Activity of 57Z mutants from bacteria. Transcription activity of purified 57Z was compared with those of ZΔE, 57ZA, and 57ZF (top three panels) using Z7CAT as the reporter and the range of protein concentrations indicated. Transcription assays contained 500 ng of Z7CAT (except for 57ZF, 50 ng of Z7CAT), and transcripts were detected by primer extension as described for Figure 3. A single experiment with different mutants is also shown (panel second from the bottom). 57Z, ZbZ, and 57ZA proteins were also tested on a reporter (RSVCAT) which does not contain zta binding sites (bottom panel).

mammalian 57Z in vivo (*10, 17*). Transcription was monitored by primer extension of CAT transcripts using labeled primer and under a range of reaction conditions (Figure 3). Z7CAT alone (500 ng) gave only a low level of activity and was activated by increasing amounts of 57Z, with activity peaking at ~250−500 ng of protein. Activity of 57Z was maximal at 500 ng of Z7CAT DNA template (data not shown) but could readily be detected using only 50 ng of template (Figure 3). DNA templates containing one (Z1CAT) or three (Z3CAT) zta binding sites were negligibly or moderately activated by 57Z, respectively, demonstrating that activation by 57Z requires zta binding sites. To assess the efficiency of transcriptional activation by 57Z, we made comparisons with two systems known to exhibit very high levels of transcription activity in vitro (Figure 3). 57Z activates Z7CAT with an efficiency comparable to that of activation of the adenovirus E4 promoter by endogenous ATF1/CREB (*32*) or activation of a Gal4-driven promoter (G4CAT) by a Gal4VP16 fusion protein produced in bacteria (*35*). These results show that 57Z activates transcription very effectively in vitro.

To assess the critical issue of EAD dependence, different mutant proteins were tested for transcriptional activity (Figure 4). Deletion of EAD1−57 significantly

reduced activity (compare 57Z with ZΔE), further deletion of the ATF1 portion had no effect (compare ZΔE with ZbZ), and the zta bZIP domain alone (ZbZ) exhibited a small amount of residual activity. Thus, EAD1−57 contributes to the transcriptional activity of 57Z. Consistent with the ability of Phe to substitute for Tyr in vivo (*10*), 57ZF retained activity in vitro (Figure 4). Surprisingly, however, 57ZA [with Tyr to Ala substitutions that eliminate activity in vivo (*10*)] was not defective and in fact had moderately higher activity than 57Z. We conclude that although EAD1−57 has high activity in vitro, the effect of specific mutations within EAD1−57 in vitro does not correspond with that observed in vivo (*10*).

*In Vitro Activity of Mammalian EAD-Containing Proteins.* In light of the results described above, we sought to obtain 57Z from mammalian cells. In our experience, it has proven to be difficult to express and purify affinity-tagged EAD-containing proteins from insect virus systems or indeed from mammalian cells, due to relatively low levels of expression and very poor yields (this was problematic even for proteins produced in bacteria). However, the high sensitivity of the in vitro trancription assay [note that as little as 15 ng of 57Z can be assayed using Z7CAT (Figure 3)] suggested that it might be feasible to obtain sufficient test proteins from transfected mammalian cells. Preliminary tests indicated that only limited amounts (~4 μL or 10% by reaction volume) of total cell extracts could be added to in vitro transcription reactions without inhibition, thus necessitating partial purification and concentration of test proteins.

Intrinsically disordered proteins are generally heat stable (*36*), the EAD is natively unstructured (*10*), and ATF1 is heat stable (*29*), together suggesting that 57Z or related proteins containing an almost intact EAD [N3Z, N3ZA, and N3ZF (Figure 6)] would be heat stable and thus amenable to a simple partial purification. Indeed, following heat treatment of mammalian cell extracts containing 57Z, ZΔE, and 57ZA (data not shown) or N3Z, N3ZA, and N3ZF (Figure 6), all of the above proteins remained soluble. This enabled a simple one-step removal of the majority of unwanted proteins and also for up to 20-fold concentration of the residual soluble proteins via filter centrifugation (see Experimental Procedures).

For direct comparison with proteins from bacteria, partially purified proteins were prepared from whole cell extracts obtained from mammalian cells transfected with expression vectors for 57Z and 57ZA. Gel mobility shift assays were employed to establish functionality of the proteins and to equalize the amounts added to in vitro transcription assays (Figure 5). Transcription reactions were performed as described for bacterial proteins, using Z7CAT as the reporter and the adenovirus major late promoter (MLP) as a control (Figure 5). Mammalian 57Z exhibited only minimal activation of Z7CAT (and is therefore less active than bacterial 57Z), while mammalian 57ZA activated to much higher levels. We conclude that similar to the effect observed for 57Z and 57ZA proteins from bacteria, the activity of 57Z from mammalian cells is lower than that of 57ZA.

While a range of mutants has previously established 57Z as an appropriate in vivo model for the intact EAD (*10, 17*), we considered one remaining possibility, namely that the small size of the EAD region in 57Z (EAD1−57) might somehow compromise bona fide EAD function in vitro. We

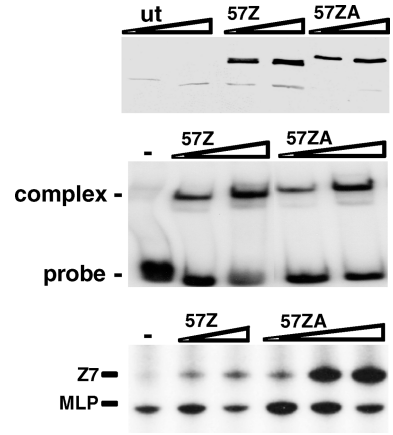


FIGURE 5: In vitro transcription using 57Z from mammalian cells. Partially purified 57Z and 57ZA were prepared from mammalian whole cell extracts (see Experimental Procedures) and quantified by Western blotting of KT3 epitope-tagged proteins (top). ut denotes the control fraction from untransfected cells. The DNA binding activity of partially purified proteins (middle) was determined by gel mobility shift assays in the responsive range (as described in the legend of Figure 2). In vitro transcription assays using different amounts of test protein, Z7CAT reporter, and the adenovirus major late promoter (MLP) as a control were performed as described for Figure 3. Z7CAT and MLP transcripts were detected by primer extension using labeled CAT or MLP primers (bottom).

therefore sought to test proteins containing much larger regions of the EAD [N3Z, N3ZA, and N3ZF (Figure 6)]. This was not possible using whole cell extracts due to the relatively lower level of expression of N3Z and N3ZF. We were, however, able to prepare sufficient concentrations of N3Z and N3ZF and control protein N3ZA [as indicated by gel mobility shift assays (Figure 6)] by using nuclear extracts and heat denaturation and concentration (as described in Experimental Procedures).

As was the case for 57Z from mammalian cells, N3Z only weakly activated Z7CAT, while in contrast, N3ZA activated Z7CAT to very high levels (Figure 6). Surprisingly, N3ZF was also very active (similar to N3ZA), and the reason for the difference in activity between N3Z and N3ZF is not clear (see Discussion). In summary, for all in vitro assays employed, the activity of proteins that are active in vivo (57Z, 57ZF, N3Z, and N3ZF) is, in every case, either less than or equal to the activity of proteins (57ZA and N3ZA) that are strongly defective in vivo (*10*). Thus, using an established methodology for testing mammalian activator proteins, our results show that there is a discrepancy between the transcriptional properties of the EAD in vitro versus in vivo.

## DISCUSSION

A previous study reported on the transcriptional activity of EFPs in vitro (*9*) and demonstrated modest activity for EWS/FLI1. Notably, ref *9* did not evaluate the contribution of the EAD to EWS/FLI1 activity, and thus, we describe, for the first time, the transcriptional properties of the EAD in vitro. We demonstrate that the in vitro activity for certain EAD-containing proteins is very potent. However, the discordant effect of EAD mutations in vivo (*10*) versus that observed in vitro suggests that crude soluble extracts do not support bona fide EAD activity.

The possibility that the activity we describe (for N3ZF from mammalian cells or 57Z from bacteria) may reflect authentic

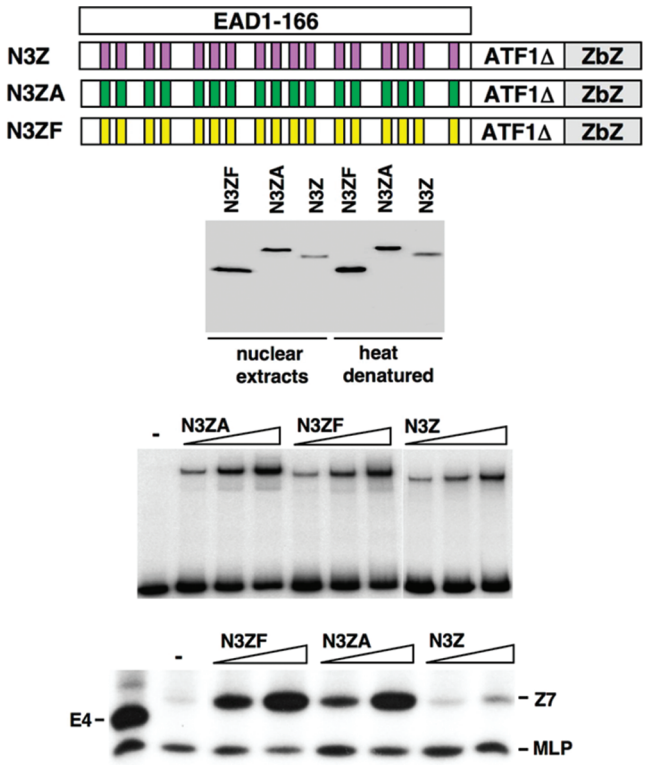


FIGURE 6: Activity of proteins containing a longer region of the EAD. N3Z contains residues 1−166 of the EAD but is otherwise identical to 57Z. Several tyrosine residues (purple boxes in N3Z) that together are critical for transcriptional activation in vivo (*10*) are shown. N3ZA and N3ZF are identical to N3Z except that the tyrosine residues highlighted are all changed to Ala (N3ZA, green boxes) or Phe (N3ZF, yellow boxes). Partially purified N3Z, N3ZA, and N3ZF were prepared from nuclear extracts (see Experimental Procedures). A Western blot of KT3 epitope-tagged proteins (Top) in nuclear extracts and soluble proteins following heat denaturation is shown. The DNA binding activity of partially purified proteins (middle) was determined by gel mobility shift assays (as described in the legend of Figure 2). In vitro transcription assays (bottom) using different amounts of test protein, Z7CAT reporter, and the adenovirus major late promoter (MLP) as a control were performed as described for Figure 3.

EAD activity cannot be ruled out, but we must account for some anamolous results. First, the fact that proteins with Tyr to Ala substitutions (57ZA and N3ZA) are not defective in vitro (and are even hyperactive) might be explained by gratuitous creation of an activation domain that functions in vitro and that masks the effect of Tyr mutation. Second, proteins from mammalian cells that have low activity in vitro (57Z and N3Z) might undergo post-translational modifications that inactivate the EAD in vitro. Such putative inhibitory effects would (according to our results) be circumvented by production of 57Z in bacteria or by substitution of Tyr with Phe (in N3ZF) in mammalian cells. One possibility is that extensive Tyr and Ser phosphorylation of the EAD in vivo (*20*) might allow promiscuous binding of putative inhibitory proteins in crude nuclear extracts. Given the large number of proteins that may bind to the EAD (*12, 13*), the notion that some would be inhibitory to a particular EAD function in vitro (but not in vivo) seems quite plausible.

The possibility that improper protein folding might compromise the function of 57Z and N3Z (but not N3ZF) is unlikely. The proteins being studied are all functional for DNA binding (and thus are not globally misfolded) and tend toward native disorder with some residual structure (*10*). In contrast to globular proteins, intrinsically disordered proteins may actually gain structure at elevated temperature due to enhanced hydrophobic interactions (*37*). Such transitions are usually reversible, however (*37*), suggesting that, in relation to our experiments, heat treatment is not expected to alter the structure or activity of 57Z or N3Z.

If the EAD-dependent activity that we have detected (for N3ZF or bacterial 57Z) is not authentic, then our results need be considered in a different light. The EAD resembles other transcriptional activation domains (TADs) (*38*) and contacts several transcriptional components (*39*−*41*), suggesting that EFPs act directly in transcription complexes. Potent activation by TADs often involves synergism with multiple coactivators (*42*), and the extended and flexible EAD may well act in this manner via highly synergistic functional elements (*17*) and potential interaction with numerous proteins (*12*). Improper EAD function in vitro could readily be explained if key coactivators are deficient or inactivated in cell extracts.

Unfavorable reaction kinetics could also impact EAD function in vitro, and it should be noted in this connection that transcription in nuclear extracts is relatively inefficient (*43*). Artificial promoter recruitment of preinitiation complex (PIC) components or histone acetyl transferase (HAT) in vivo (*44*) does not bypass the requirement for the EAD, suggesting that the EAD acts following PIC assembly and perhaps on elongation (*44*). Strong synergistic activation by the EAD in vivo might reflect stimulation of multiple steps in transcription (including PIC formation, promoter clearance, and transcript elongation), and perhaps the rate-limiting steps or cofactor concentrations prevailing in crude nuclear extracts preclude stimulation by the EAD.

A remaining possibility is that, as is often the case for activator-dependent transcription (*45, 46*), the EAD may function only on chromatin DNA templates. There is circumstantial evidence that may point in this direction. The EAD binds to the coactivator CREB-binding protein (CBP/p300) (*39, 47*) which is implicated in chromatin remodeling and serves as a critical coactivator for several chromatin-dependent activators (*48*−*51*). In addition, many chromatin organizing proteins resemble the EAD in that they are generally disordered with some residual structure (*52*) and thus belong to the pre-molten globule class of disordered proteins.

To conclude, our findings suggest that development of a cell-free transcription assay for the EAD will depend on several advances, most likely including identification of functionally relevant EAD-interacting proteins together with employment of more sophisticated transcription systems, possibly with purified components (*53*).

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