Okadaic Acid-mediated Induction of the c-fos Gene in Estrogen Receptor-negative Human Breast Carcinoma Cells Utilized, in Part, Posttranscriptional Mechanisms Involving Adenosine-Uridine-rich Elements

Lulu Farhana, Madanamohan Boyanapalli, Sheng-Hung R. Tschang, Rong-Juan Sun, C. K. Alex Hsu, Yu-Xiang Zhang, Joseph A. Fontana, and Arun K. Rishi

John D. Dingell Veterans Affairs Medical Center, Department of Internal Medicine and Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

Abstract

Signal transduction via modulation of phosphorylation after selective inhibition of protein phosphatase (PP) 1 and/or PP2A appears to play a role in okadaic acid (OA)-mediated effects. Treatment of several estrogen receptor-negative human breast carcinoma (HBC) cells with 100 nm OA resulted in induction of c-fos, c-myc, and cyclin-dependent kinase inhibitor p21\textsuperscript{WAF1/CIP1} genes. Transfections of various luciferase reporter constructs in HBC cells revealed involvement of activator protein-1-dependent as well as -independent pathways in induction of the c-fos gene by OA. MDA-MB-468 HBC cells were stably transfected with plasmids expressing luciferase, chimeric luciferase- c-fos 3’ untranscribed region (3’ UTR), or chimeric luciferase-p21\textsuperscript{WAF1/CIP1} 3’ UTR mRNAs. Expression of chimeric luciferase-c-fos and luciferase-p21\textsuperscript{WAF1/CIP1} mRNAs was elevated by OA in several independent sublines. Actinomycin D chase experiments revealed an enhanced rate of decay of luciferase-c-fos mRNA, whereas treatment with OA caused –3.5-fold enhanced stability of the chimeric luciferase-c-fos mRNA only. By transfecting different plasmids containing deletions of c-fos 3’ UTR, OA-responsive sequences were mapped to an 86-nucleotide, AU-rich region. UV cross-linking experiments using HBC cell cytosolic proteins showed multiple complexes with the AU-rich region subfragments of c-fos, as well as c-myc and p21\textsuperscript{WAF1/CIP1} mRNAs. OA enhanced binding of a novel M₇ –75,000 protein present in the cytosolic extracts of HBC cells to the AU-rich RNA probes of all of the above three genes. Taken together, OA regulation of HBC cell gene expression involves the activator protein-1 pathway, as well as enhanced binding of a novel M₇ –75,000 protein to an AU-rich region of the 3’UTRs of the target genes.

Introduction

OA\textsuperscript{3} is a polyether compound of a C₃₈ fatty acid that belongs to a new category of tumor-promoting agents that do not activate protein kinase C, yet can cause an increase in the phosphorylation of cellular proteins because of its inhibition of PP1 and PP2A (1). Tumor-promoting effects of OA are presumably attributable to increased phosphorylation of one or more proteins that serve as substrates for protein kinase C and are dephosphorylated by PP1 and/or PP2A. Numerous studies have shown the usefulness of OA in understanding novel cellular processes that are regulated by phosphorylation (2).

OA regulates gene expression at transcriptional, posttranscriptional, or posttranslational levels. For example, exposure of human skin fibroblasts to OA resulted in transcriptional repression of elastin (3) and type I and type III collagen genes (4), whereas several genes including collagenase, stromelysin (Ref. 4 and references within), c-Jun, Egr, and IL-6 (Ref. 5 and references within) were transcriptionally induced in human skin fibroblasts in the presence of OA. IL-8 gene induction in the presence of OA involves both transcriptional (via a nuclear factor-κB site in the promoter of the IL-8 gene) as well as posttranscriptional (enhancement of stability of the IL-8 message) mechanisms in the HL-60 cells (6). Similarly, OA-dependent enhanced expression of nerve growth factor gene in the rat cortical astrocytes has also been shown to be both the transcriptional as well as posttranscriptional levels (7), whereas OA treatment of mouse P19 embryonal carcinoma cells leads to reduced stability of neurofilament L gene mRNA (8). In NIH3T3 cells, OA has been shown to cause increased phosphorylation of p53 (9), whereas OA-mediated inhibition of phosphorylation of retinoblastoma gene product was noted in the mouse embryonic fibroblasts (10), and OA also caused repression of expression and kinase activities of the cell cycle regulatory proteins cdc2 and cyclin A (11). OA-regulated cyclin-dependent kinase inhibitor p21\textsuperscript{WAF1/CIP1} gene at both the transcriptional (via the AP-2 site in its promoter) as well as post-
Okadaic Acid-mediated Induction of the c-fos Gene

transcriptional levels in K549 cells (Ref. 12 and references within). Transcription of several other genes, including heat shock protein 70 and manganese superoxide dismutase, was induced by OA in N-18 mouse neuroblastoma cells and HeLa cells, respectively (13, 14). OA also caused induction of the cellular proto-oncogene c-fos in different cell types including MCF-7 HBC cells, where it has been shown to involve transcriptional as well as posttranscriptional mechanisms (15, 16).

The present study was undertaken to further understand the molecular basis of OA-dependent gene regulation with reference to the ER-negative HBC cells. Cyclin-dependent kinase inhibitor p21WAF1/CIP1 and cellular proto-oncogenes c-fos and c-myc expression was elevated in OA-treated HBC cells. We found that OA used transcriptional mechanisms involving the AP-1 pathway, as well as posttranscriptional mRNA stabilization mechanisms. An AU-rich, OA-responsive, 86-nucleotide subfragment of c-fos 3′ UTR was mapped by deletion analyses. UV cross-linking and gel mobility shift experiments using radiolabeled ARE subfragment probes of c-fos, as well as p21WAF1/CIP1 and c-myc 3′ UTRs, revealed the presence of multiple RNA-protein complexes. Binding of a novel M₀ ≈ 75,000 protein to ARE subfragment probes was significantly elevated in cytosolic extracts derived from OA-treated HBC cells. We propose that elevated binding of the putative M₀ ≈ 75,000 protein to the AREs of 3′ UTRs constitutes one of the key mechanism(s) involved in posttranscriptional regulation of gene expression by OA in the HBC cells.

Results

OA Exposure Caused Induction of Expression of Several Genes in ER-negative Breast Carcinoma Cells. MDA-MB-468 and MDA-MB-435 HBC cells were exposed to 10-, 20-, 40-, 60-, 80-, and 120-nM doses of OA for a period of 7 h. Total RNAs were prepared from both the floating and adherent cells, and expression of c-fos, c-myc, and p21WAF1/CIP1 genes was evaluated by Northern blot hybridization. As shown in Fig. 1, the 120-nM dose of OA caused elevated expression of the c-fos, p21WAF1/CIP1, and c-myc genes in the HBC cells.

OA Caused Transcriptional Up-Regulation of Several Genes. To investigate mechanism(s) of gene regulation in the presence of OA, different luciferase reporter gene constructs (Fig. 2) were transiently transfected in HBC cells. The plasmids containing p21WAF1/CIP1 promoter (WWP-Luc; Ref. 12), GADD45 promoter (GADD-Luc 6.3; Ref. 17), and c-fos promoter (Fos-Luc1) were used in the first instance, because all of the three genes were elevated in the presence of OA (see Fig. 1 and data not shown). As shown in Fig. 3, A and B, treatment with OA caused 2–3-fold increase in luciferase activities in the case of plasmids containing the GADD45 promoter and c-fos promoter. The plasmid WWP-Luc- containing promoter for the p21WAF1/CIP1 gene, however, showed 2-fold enhancement of luciferase activity in the presence of OA only in MDA-MB-435 HBC cells. Because AP-2 elements present in the promoter of the p21WAF1/CIP1 gene have been demonstrated previously to be involved in the OA-dependent induction of p21WAF1/CIP1 in A549 cells (12), it would appear that additional, perhaps novel, mechanisms exist for OA-dependent induction of the p21WAF1/CIP1 gene in HBC cells.

OA activates AP-1 complexes and AP-1-driven promoters of the target genes including c-fos (Ref. 18 and references within). Because OA caused enhanced expression of the c-fos gene in the ER-negative HBC cells, the possibility of the involvement of AP-1-dependent as well as -independent pathways was further investigated. The plasmids Fos-Luc1, Fos-Luc2.5, and Fos-Luc3.1 were independently and transiently transfected into MDA-MB-468 HBC cells. OA (100 nM) was added 40 h after transfection; both the controls and treated cultures were harvested at 48 h and assayed for luciferase activities. All of the constructs elicited an 2.5–3-fold increase in the luciferase activities in the presence of OA (Fig. 3C). In addition, Fos-SV40-Luc clone 4.12 and pGL2- Promoter plasmids were independently and transiently transfected into MDA-MB-468 HBC cells, followed by treatment of cells with 100 nM OA as described above. Fos-SV40-Luc clone 4.12 did not elicit OA-dependent enhancement of luciferase activities when compared with the pGL2-Promoter construct (data not shown).

Fig. 1. Dose-dependent induction of c-fos, c-myc, and p21WAF1/CIP1 mRNAs in the presence of OA in HBC cells. MDA-MB-468 and MDA-MB-435 HBC cells were treated with 0, 10, 20, 40, 60, 80, and 120 nM OA for a period of 7 h, followed by RNA extraction and Northern blot hybridization with radiolabeled c-fos, c-myc, and p21WAF1/CIP1 cDNA probes as described in “Materials and Methods.” Northern blots were washed twice in 1 × SSC, 0.1% SDS at 65°C for 30 min each time. Ethidium bromide stain of the RNA gels is also shown to assess RNA loading, as indicated by the signals for 28S and 18S rRNAs.
independent pathways. Furthermore, the plasmid containing AP-1 elements as well as the entire 3'UTR of the c-fos mRNA ([AP-1]5-CMV-Luc-Fos) elicited a 5–6-fold increase in luciferase activities in the presence of OA (Fig. 3D), suggesting that pathways using AP-1 elements and 3'UTR sequences cooperate in OA-mediated induction of the c-fos gene in HBC cells. In addition, as shown in Fig. 3E, the plasmid CMV-Luc-Fos 5A.7 showed reduced activities in the presence of OA when compared with plasmid CMV-Luc-Fos 5.2, indicating that the cis elements present in 8-kbp 3'UTR fragment involved in OA-dependent up-regulation of the c-fos gene were functional in their sense orientation. Thus, the OA-responsive sequences of c-fos 3'UTR are unlikely to be transcriptional in their function, because sequences mediating transcriptional repression or enhancement of a given gene are known to function independently of their orientation and location from the transcription start site. The plasmid CMV-Luc clone 6.1 also elicited a 2-fold increase in the luciferase activity in the presence of OA when
compared with untreated controls, suggesting that OA indeed used multiple mechanisms for up-regulation of different genes in the HBC cells. The OA-responsive cis sequences present in the c-fos 3' UTR were further mapped by using various deletion constructs (see Fig. 2). OA caused enhanced luciferase activities of all of the constructs except CMV-Luc-6.1 and CMV-Luc-Fos-9.11 (Fig. 3), suggesting that OA-responsive elements were located within the 86-nucleotide ARE of the 3' UTR of the c-fos gene. Thus, in addition to AP-1 transactivation, the data in Fig. 3 demonstrate that cis elements encompassed in the 3' UTR of the c-fos gene were used in the OA-dependent regulation of the c-fos gene in the ER-negative HBC cells.

OA Caused Posttranscriptional Regulation of c-fos and p21WAF1/CIP1 Genes. To further elucidate OA-mediated posttranscriptional regulatory mechanism(s), MDA-MB-468 HBC cells were transfected with either CMV-Luc-6.1 or CMV-Luc-Fos-9.11 (Fig. 3F), suggesting that OA-responsive elements were located within the 86-nucleotide ARE of the 3' UTR of the c-fos gene. Thus, in addition to AP-1 transactivation, the data in Fig. 3 demonstrate that cis elements encompassed in the 3' UTR of the c-fos gene were used in the OA-dependent regulation of the c-fos gene in the ER-negative HBC cells.

Fig. 3. AP-1-dependent and -independent induction of c-fos gene by OA. MDA-MB-468 HBC cells (A, C, D, E, and F) or MDA-MB-435 HBC cells (B and G) were transiently transfected with different luciferase reporter gene plasmids in combination with CMV-b-gal plasmid. Approximately 40 h after transfections, the cells were then treated with 100 nM OA for an additional 7 h, and luciferase and b-galactosidase activities were measured as described in “Materials and Methods.” The luciferase activities were expressed as light units and normalized to b-galactosidase activities expressed as absorbance. Columns represent the means of three independent experiments expressed relative to the luciferase activities obtained from untreated controls, which were arbitrarily defined as 1; bars, SE.

expression of chimeric luciferase-c-fos (Lanes 1 and 3) as well as luciferase-waf (Lanes 9 and 11) mRNAs was reduced when compared with the expression of luciferase mRNA (Lanes 5 and 7). Thus, the presence of the 3'UTRs of the c-fos and p21WAF1/CIP1 genes down-regulated expression of the CMV-Promoter-driven luciferase reporter gene in the HBC cells. Furthermore, expression of chimeric luciferase-c-fos (Lanes 2 and 4) and luciferase-waf (Lanes 10 and 12) mRNAs was elevated by ~6–7-fold in the cells treated with OA (Fig. 4). Taken together, the data in Fig. 4 suggested that 3'UTR subfragments of c-fos and p21WAF1/CIP1, present in plasmid clones CMV-Luc-Fos-5.2 and CMV-Luc-WAF-4.2, contained OA-responsive cis-regulatory sequences.

Two independent MDA-MB-468 HBC cell sublines derived after transfection of plasmids CMV-Luc-6.1 or CMV-Luc-Fos-5.2 were incubated in the presence of OA for 5 h. Transcriptional inhibitor AMD (4 µg/ml) was then added to the cells, and luciferase mRNA levels were determined at various time intervals. The mRNA decay plotting was carried out as we have described before (17). Fig. 5 shows that the presence of OA enhanced the stability of the chimeric luciferase-c-fos mRNA by ~3.5-fold in the HBC cell sublines derived from transfection of plasmid CMV-Luc-Fos-5.2 (t1/2 of ~75 min for control versus t1/2 of ~270 min for OA-treated cells).
OA Modulated HBC Cell Cytosolic Protein Interactions with AREs of c-fos mRNA. Different RNA binding proteins have been shown previously to interact with AREs of several mRNAs including c-fos and p21\(^{WAF1/CIP1}\) (20–23). The RNA-protein interactions were evaluated using a radiolabeled RNA subfragment containing AREs belonging to c-fos (plasmid pBSK-Fos 10.1; Fig. 2) in conjunction with OA-treated and untreated HBC cell cytosolic protein extracts. Several HBC cell cytosolic proteins interacted with c-fos ARE (Fig. 6A). These include four major complexes of approximately Mr 37,000–40,000, Mr 51,000, Mr 75,000, and Mr 118,000 sizes (Fig. 6A, Lanes 2 and 5). The presence of an ~200-fold excess of an unlabeled c-fos ARE RNA abolished binding of the above cytosolic proteins to the c-fos probe (Fig. 6A, Lanes 3 and 6). However, the presence of an ~200-fold molar excess of a nonspecific, unlabeled RNA derived from plasmid pBSK-GADD 12.4 (see Fig. 2) failed to compete for any RNA-protein binding (Fig. 6A, Lanes 4 and 7), suggesting that the binding of the above cytosolic proteins to the radiolabeled ARE probe of c-fos 3’UTR was specific. The RNA-protein interactions using cytosolic proteins derived from OA-treated HBC cells showed elevated binding of the above noted Mr 38,000–40,000 complex as well as the Mr 75,000 complex with the c-fos ARE probe, whereas the binding of the putative Mr 118,000 complex was reduced (Fig. 6A, Lanes 5 and 7).

The nature of the cytosolic proteins interacting with the ARE of c-fos 3’UTR was further evaluated. As shown in Fig. 6B, pretreatment of extracts with proteinase K (Lanes 3 and 6) or alkaline phosphatase (Lanes 4 and 7) abrogated RNA-protein interactions, indicating that different proteins binding with c-fos 3’UTR AREs were phosphoproteins.

OA Caused Elevated Binding of a Novel Mr ~75,000 HBC Cytosolic Protein to the AU-rich Sequences of Different Target Genes. The interactions of the cytosolic proteins derived from OA-treated HBC cells with the probes con-
Okadaic Acid-mediated Induction of the c-fos Gene

Fig. 6. Analysis of RNA-protein interactions by gel mobility shift assays. Binding of HBC cell cytosolic proteins to 32P-labeled c-fos ARE sense strand riboprobe. Transcription and labeling of the probes and the binding reactions were as described in “Materials and Methods.” A, Lane 1, probe only; Lane 2, probe and the cytosolic protein extracts; Lane 3, probe, cytosolic protein extracts, and 200-fold excess of the cold unlabeled probe fragment RNA; Lane 4, probe, cytosolic protein extracts, and 200-fold excess of the cold unlabeled nonspecific 45-nucleotide-long sense strand human GADD45 5' UTR RNA fragment (17); Lane 5, probe and cytosolic protein extracts from OA-treated cells; Lane 6, probe, cytosolic protein extracts from OA-treated cells, and 200-fold excess of the cold unlabeled probe fragment RNA; Lane 7, probe, cytosolic protein extracts from OA-treated cells and 200-fold excess of the cold unlabeled nonspecific 45-nucleotide-long sense strand human GADD45 5' UTR RNA fragment (17). Binding of HBC cell cytosolic proteins to the 32P-labeled c-fos ARE probe and the cytosolic protein extracts from OA-treated cells and calf intestinal alkaline phosphatase (40 units).

Fig. 7. Binding of HBC cell cytosolic proteins to the 32P-labeled ARE containing 3'UTRs of p21WAF1/CIP1, c-fos, and c-myc genes as well as the 20-nucleotide URE subfragment of c-fos 3'UTR. Transcription and labeling of the probes and the binding reactions were as described in “Materials and Methods.” A, Lane 1, probe only; Lane 2, probe and the cytosolic protein extracts; Lane 3, probe, cytosolic protein extracts, and 200-fold excess of the cold unlabeled probe fragment RNA; Lane 4, probe, cytosolic protein extracts, and 200-fold excess of the cold unlabeled nonspecific 45-nucleotide-long sense strand human GADD45 5' UTR RNA fragment (17); Lane 5, probe and cytosolic protein extracts from OA-treated cells; Lane 6, probe, cytosolic protein extracts from OA-treated cells and 200-fold excess of the cold unlabeled probe fragment RNA; Lane 7, probe, cytosolic protein extracts from OA-treated cells and 200-fold excess of the cold unlabeled nonspecific 45-nucleotide-long sense strand human GADD45 5' UTR RNA fragment (17). B, Lane 1, c-fos ARE probe only; Lane 2, c-fos ARE probe and the cytosolic protein extracts; Lane 3, c-fos ARE probe and cytosolic protein extracts from OA-treated cells; Lane 4, c-fos ARE probe, cytosolic protein extracts from OA-treated cells, and 200-fold excess of the cold unlabeled c-fos ARE probe fragment RNA; Lane 5, c-fos URE probe only; Lane 6, c-fos URE probe and cytosolic protein extracts; Lane 7, c-fos URE probe and cytosolic protein extracts from OA-treated cells; Lane 8, c-fos URE probe and cytosolic protein extracts from OA-treated cells and 200-fold excess of the cold unlabeled c-fos ARE probe fragment RNA; Lane 9, c-myc 3'UTR probe only; Lane 10, c-myc 3'UTR probe and the cytosolic protein extracts; Lane 11, c-myc 3'UTR probe and cytosolic protein extracts from OA-treated cells; Lane 12, c-myc 3'UTR probe, cytosolic protein extracts from OA-treated cells, and 200-fold excess of the cold unlabeled c-myc ARE probe fragment RNA. * and **, locations of putative M, 118,000 and M, 75,000 complexes, respectively.
abolished binding of the above cytosolic proteins to either c-fos (Lane 4), URE (Lane 8), or c-myc (Lane 12) probes. The cytosolic proteins derived from OA-treated HBC cells showed elevated binding of the above noted M, 38,000–40,000 complex with all of the above three probes (Fig. 6B, Lanes 3, 7, and 11). However, as noted before in Figs. 6A and 7A, the binding of the M, 75,000 and M, 118,000 proteins present in the OA-treated HBC cell extracts was modulated in the case of c-fos and c-myc RNA probes but not when URE probe was used (Fig. 7B, compare Lanes 3 and 11 with Lane 7). The data in Fig. 7, therefore, strongly suggest that OA-dependent elevated RNA-protein interactions involving the putative M, 75,000 protein with the c-fos, p21WAF1/CIP1, and c-myc AU-rich RNAs consisted of novel protein that was different than the protein observed in the M, −75,000 complex noted with the URE probe. Furthermore, the binding of the M, 118,000 complex was also noted with all of the probes used in Fig. 7. However, reduced binding of the putative M, 118,000 complex was noted in the case of cytosolic extracts derived from OA-treated HBC cells when either c-fos or c-myc (Fig. 7B, Lanes 3 and 11) ARE probes were used but not when URE probe was used (Fig. 7B, Lane 7).

Discussion

In the present investigation, we found that submicromolar concentrations of OA caused elevated expression of several mRNAs including p21WAF1/CIP1, c-myc, and c-fos in HBC cells. OA also caused induction of GADD45 and early-response genes c-jun, egf-1, and NAK1. The elevated expression of c-fos, p21WAF1/CIP1, and GADD45 by OA was also noted at the individual protein levels (data not shown). Both the ER-negative MDA-MB-231 and MDA-MB-468 HBC cells possess a mutant, nonfunctional p53 (25). Thus, OA-dependent up-regulation of p53-responsive p21WAF1/CIP1 and GADD45 genes in various HBC cells used in this study further corroborates the earlier observation (16) that wild-type p53 function was not necessary for the mechanism(s) of OA-dependent gene regulation.

The upstream promoter of the c-fos gene has been shown to contain several regulatory sequences including SRE, sis-inducible element, Ets motif adjoining SRE, and AP-1/ATF sites in the proximity of SRE (Ref. 26 and references within). The cellular retinoblastoma susceptibility gene protein Rb also binds to specific cis elements in the c-fos gene promoter, leading to repression of c-fos transcription (27). We found that OA induction of c-fos gene was mediated, in part, by the elements present in the c-fos promoter, and given the similar levels of enhancement of (AP-1) 5-CMV-Luc activities by OA (see Fig. 3), it was likely that OA activation of the c-fos promoter involved AP-1 cis-trans interactions.

In this report, we present the evidence, for the first time, implicating the role of 3′UTR in induction of the c-fos gene in the HBC cells. We demonstrate that OA caused significant posttranscriptional stabilization of chimeric luciferase mRNAs containing entire 3′UTR sequences of the c-fos gene. Deletion mapping revealed that the ARE of the c-fos 3′UTR mediated, in part, OA-dependent induction of the c-fos gene in HBC cells (see Fig. 9). AREs (5′-AUUUA-3′) located in the 3′UTR have been shown previously to regulate expression of several genes including c-fos, c-myc, and p21WAF1/CIP1 at posttranscriptional levels (Refs. 23 and 28 and references within). Indeed, the presence of c-fos 3′UTR caused reduced levels of the chimeric luciferase-c-fos as well as chimeric luciferase-p21WAF1/CIP1 mRNAs, when compared with the luciferase mRNA expressed in the different HBC sublines with stably integrated plasmids CMV-Luc-Fos 5.2, CMV-Luc-WAF 4.2, or CMV-Luc 6.1.

Recently, ARE binding proteins from several labile mRNAs and different cell types have been characterized. For example, a M, 32,000 protein present in both nuclear and cytosolic extracts of HeLa cells has been shown to bind ARE of different labile mRNAs including c-fos (20). Another M, 37,000–40,000 size ARE binding protein (AUF-1) has also been shown to be present in both the nuclear and cytosolic extracts of K562 cells (29). In addition, heterogeneous nuclear ribonucleoproteins A1 and C of M, 36,000 and M, 43,000 size, respectively, and the M, 30,000 inducible factor called AU-B, have been shown to bind AREs and were found to be cytoplasmic in nature (30, 31). Furthermore, several Elav-like proteins have been demonstrated to bind AU-rich elements, and a M, 36,000 protein named HuR binds AREs of c-fos mRNA and can be induced to redistribute from nucleus to cytosol (21, 23). Indeed, several of the nuclear proteins derived from OA-treated HBC cells belonging to a size range of M, 35,000–60,000 showed elevated binding to the c-fos ARE probe obtained from plasmid pBSK-Fos 10.1 (data not shown). Whether the ARE binding HBC cell nuclear proteins ranging from M, 35,000–60,000 size consist of novel and/or the previously identified RNA binding nuclear proteins, including heterogeneous nuclear ribonucleoproteins (31), AU-B (30), and Elav-like proteins (23), along with their precise role in OA-dependent mRNA stabilization in HBC cells, remains to be determined. Nevertheless, similar to the data shown in Figs. 6 and 7, M, −75,000 protein complex also demonstrated elevated binding to the c-fos ARE probe when nuclear extracts derived from OA-treated HBC cells were used (not shown).

Thus, the underlying conservation and differences observed in this study among the HBC cell cytosolic and nuclear proteins interacting with c-fos ARE probe may point to the existence of multiple levels of gene regulation by OA. Nevertheless, the HBC cell ARE binding proteins were found to be phosphoproteins. Because OA treatment has been shown previously to enhance phosphorylation of cellular proteins (15), it is possible that OA-dependent modulation of phosphorylation of the HBC cell ARE binding proteins may constitute, in part, the basis for induction of c-fos, c-myc, and p21WAF1/CIP1 genes.

The requirement for ARE in the OA-dependent stabilization of c-fos was highlighted when a 20-nucleotide URE sequence of c-fos 3′UTR failed to show OA-dependent elevated binding of the putative M, 75,000 HBC cell cytosolic protein. It should, however, be noted that the above 20-nucleotide URE has been shown previously to bind, in vitro, with three complexes C1, C2, and C3 having RNA-binding proteins of M, 37,000, M, 65,000–71,000, and M, 85,000 sizes, respectively (21). We did not find elevated binding of the M, 65,000–71,000 or M, 85,000 complexes to the similar 20-nucleotide URE probe when cytosolic extracts obtained from OA-treated HBC cells were used (see Fig. 7B). Therefore, the OA-dependent induction of different genes in HBC cells may use interactions of novel and/or known proteins with the OA-responsive 3′UTR sequences of the target genes in a fashion independent of the involvement of URE.
In conclusion, we report the existence of multiple mechanisms for OA-dependent regulation of the early-response gene \( c-fos \), the oncogene \( c-myC \), and the cell cycle-related genes \( p21^{WAF1/CIP1} \) and \( GADD45 \) in the ER-negative HBC cells. The evidence, including the data in this report, suggests that transcriptional mechanisms underlying OA-mediated regulation of HBC cell gene expression involve, in general, AP-1-dependent pathways. OA also uses additional posttranscriptional mechanisms of gene regulation by modulating half-lives of the target mRNAs. The posttranscriptional control mechanisms involve specific RNA-protein \( cis \)-\( trans \) interactions including elevated binding of a novel \( M_\text{r} \sim 75,000 \) cytosolic protein to the AREs in the \( 3' \) UTRs of the target genes.

**Materials and Methods**

**Materials.** DMEM, Ham’s F-12 medium, and fetal bovine serum were purchased from Life Technologies, Inc. (Grand Island, NY). The oligonucleotides for PCR amplification (see below) were purchased from either Bio-Synthesis, Inc. (Lewisville, TX) or integrated DNA Technologies, Inc. (Corvallis, OR). Amplitaq DNA polymerase and deoxynucleotides were purchased from either Bethesda Research Laboratories (Bethesda, MD) or New England Biolabs (Beverley, MA). The restriction endonucleases and the DNA modification enzymes were purchased from either Bethesda Research Laboratories (Bethesda, MD) or New England Biolabs (Beverley, MA). The reagents for dideoxy sequencing were purchased from United States Biochemical Corp. (Cleveland, OH). OA was purchased from Alexis Biochemicals (San Diego, CA).

**Plasmids and cDNA Probes.** The plasmids containing full-length human \( p21^{WAF1/CIP1} \) cDNA and human \( p21^{WAF1/CIP1} \) promoter luciferase reporter clone WWp-Luc (12) were kindly provided by Drs. Ken Kinzler and Bert Vogelstein (Johns Hopkins University, Baltimore, MD). c-fos promoter plasmid containing –356 to +109 of the human c-fos promoter (27) was obtained from Dr. Paul Robbins (University of Pittsburgh, Pittsburgh, PA), and the plasmid pcBR322 containing the entire human c-fos gene (32) was obtained from Dr. T. Curran (St. Jude’s Children Research Hospital, Memphis, TN), whereas \( AP-1_9 \)-SV40-CAT plasmid (33) was provided by Dr. D. Schadenburg (Humboldt University, Berlin, Germany).

**Cell Lines and Cell Culture.** The ER-negative MDA-MB-468 HBC cells were provided by Dr. Anne Hamburger (Greenebaum Cancer Center, University of Maryland, Baltimore, MD). The ER-negative MDA-MB-435 HBC cell line was obtained from Dr. Steven Byers (Lombardi Cancer Center, Washington, DC). The cells were cultured routinely in DMEM/Ham’s F-12 medium (1:1) supplemented with 5% fetal bovine serum as described previously (19). Cells were transiently transfected with various plasmids; \( \beta \)-galactosidase and luciferase activities were measured essentially as described previously (19). In certain cases, appropriate doses of OA were added 6–7 h prior to harvesting.

**RNA Isolation and Northern Blot Analysis.** Logarithmically growing HBC cells were treated with different doses of OA for 7 h. Expression of different genes in untreated as well as OA-treated cells was studied by Northern blot hybridization essentially as described before (19, 34).

**Cloning of Plasmid Constructs.** The \( BglII \) fragment containing five tandem repeats of the \( AP-1 \) cis element was excised from the plasmid (AP-1)\(_9\)-SV40-CAT (33) and ligated into the unique \( BglII \) site upstream of the CMV promoter in the CMV-Luc plasmid clone 6.1 (19) to obtain plasmid (AP-1)\(_9\)-CMV-Luc as per the methods described before (34). Cloning of the plasmid expressing the CMV promoter-driven chimeric luciferase-p21\(^{WAF1/CIP1}\) 3' UTR mRNA (CMV-Luc 4.2) was described previously (19). The human \( GADD45 \) gene promoter luciferase reporter construct GADD-Luc 6.3 has been described before (17).

Various c-fos Promoter-luciferase reporter clones were obtained as follows. The c-fos promoter (–0.5 kbp) and the exon 1 region from position –356 to +109 present in Fos-Luc plasmid (32) were subcloned into pGL-2Basic vector plasmid (Promega) to obtain the c-fos promoter-luciferase reporter clone Fos-Luc 1. Approximately 840 bp containing the human c-fos promoter and exon 1 region (from positions –600 to +240; Ref. 32) were PCR amplified using plasmid pc-fos(human)-1 (35) as template and oligos fos-1 (5'–CGAACAGTTCAAGTGGGCCGCTGTGAGCA-3', antisense oligo, positions 225–244; Ref. 32) and fos-2 (5’–GACGAAACGTGCTAGTATT-3’, sense oligo, positions –600 to –580; Ref. 32). It should be noted, however, that the position +240 in the above 840-bp c-fos promoter and exon 1 fragment corresponds to position +109 in the exon 1 of the previously described clone Fos-Luc (27). A three-way ligation was set up using BglII (end-filled) and HindIII-cut pGL2-Basic vector, EcoRI (end-filled) and Xhol-cut 1.3-kbp 5’UTR fragment derived from plasmid pc-fos(human)-1, and HindIII-Xhol-cut 840-bp PCR-amplified fragment of the c-fos gene to obtain the c-fos promoter-luciferase reporter clone Fos-Luc 2.5. Fos-Luc 2.5 thus contains the c-fos promoter from positions +109 to –2000 (32). Additional c-fos promoter-luciferase reporter clone Fos-Luc 3.1 was obtained by removing an –1.4-kbp, Xhol fragment of clone Fos-Luc 2.5. The c-fos promoter in the clone Fos-Luc 3.1, therefore, contains fos promoter from positions +109 to –600 (32). Approximately 2.3 kbp of NotI plus NcoI subfragment of the c-fos gene (+180 to +2531; Ref. 32) was derived from plasmid pc-fos(human)-1 (35), end-filled and cloned in the antisense orientation upstream of the SV40 promoter in the Xhol-cut, end-filled vector plasmid pGL2-Promoter (Promega) to obtain Fos-SV40-Luc clone 4.12.

Different plasmids expressing chimeric luciferase-c-fos mRNAs were generated as follows. A modified pcDNA3-luc vector clone was derived from CMV-Luc plasmid 6.1 (19), following standard manipulations to remove a –260-bp Xbal to PvuII subfragment containing Sp-6 promoter and bovine growth hormone signal sequences. –0.8 kbp of the 3'UTR immediately following the translation termination codon in exon 4 of the c-fos gene was PCR amplified using plasmid pc-fos(human)-1 (35) as template and oligos fos-3 (5’–CGACTCGAGGCCGGAAGGGGAGGCA-3’, sense oligo, positions 3723 to 3750; Ref. 32) and fos-4 (5’–CGACTCGAGGCCGGAAGGGGAGGCA-3’, antisense oligo, positions 3532 to 3549; Ref. 32). It should be noted that oligo fos-4 lies downstream of the polyadenylation signal sequences of the c-fos gene. The Xhol-cut, PCR amplified, 3’UTR fragment of the c-fos gene was cloned in the sense orientation in the unique Xhol site located at the 3’ end of the luciferase gene in the modified pcDNA3-Luc vector clone 1 above to obtain the CMV-Luc-Fos clone 5.2. In addition, the
antisense orientation at the unique positions 2937 to 2954; Ref. 32), fos-7 (5'-CGACTCGAGAAC-TACTGTGTTCCTGGC-3', sense oligo, positions 3343 to 3360; Ref. 32), or fos-9 (5'-CGACTCGAGCCTTGAGGTCTTTTGACATG-3', sense oligo, positions 3343 to 3360; Ref. 32), fos-8 (5'-CGACTCGAGTTTCCATGAAAACGTTTT-3', sense oligo, positions 3343 to 3360; Ref. 32), or fos-9 (5'-CGACTCGAGCGCTTGAGGTCTTTTGACATG-3', sense oligo, positions 3429 to 3448; Ref. 32). The resultant PCR fragments were cloned in sense orientations at the unique Xhol site of the above-described modified pcDNA3-Luc vector to obtain CMV-Luc-Fos clones 6.4, 7.1, 8.3, and 9.11. The 45-bp AU-rich subfragment of c-myc was then cloned in plasmid vector pBSKII (Stratagene) to obtain the pBSK-Fos clone 10.1. The cDNA fragment of CMV-Luc-Fos clone 8.3 was sequenced to confirm the validity of the c-myc fragment. PCR using oligo fos-4 in combination with either fos-6 (5'-CGACTCGAGAAAAACGATGGAGTGT-3', sense oligo, positions 2937 to 2954; Ref. 32), fos-7 (5'-CGACTCGAGAAC-TACTGTGTTCCTGGC-3', sense oligo, positions 3143 to 3160; Ref. 32), fos-8 (5'-CGACTCGAGTTTCCATGAAAACGTTTT-3', sense oligo, positions 3343 to 3360; Ref. 32), or fos-9 (5'-CGACTCGAGCGCTTGAGGTCTTTTGACATG-3', sense oligo, positions 3429 to 3448; Ref. 32). The resultant PCR fragments were cloned in sense orientations at the unique Xhol site of the above-described modified pcDNA3-Luc vector to obtain CMV-Luc-Fos clones 6.4, 7.1, 8.3, and 9.11. The 105-bp AU-rich subfragment of c-fos 3' UTR (positions 3343 to 3348; Ref. 32) was PCR amplified using a combination of oligos fos-8 and fos-10. Oligo fos-10 is an antisense of oligo fos-9, except that the 5' end of oligo fos-10 has an overhang for the Xbal site. The PCR-amplified, Xhol + Xbal-digested, 105-bp AU-rich subfragment was then cloned in plasmid vector pBSKII (Stratagene) to obtain pBSK-Fos clone 10.1. The cDNA fragment of CMV-Luc-Fos clone 8.3 was sequenced to confirm the validity of the c-fos insert. A 20-nucleotide-long URE (positions 3406 to 3425; Ref. 32) present in the c-fos 3' UTR was subcloned as a double stranded fragment in vector plasmid pBSK as described below. Sense and the corresponding antisense oligos, having 5' overhangs of 4 nucleotides for Xhol and Xbal sites, respectively, were synthesized. The oligos were phosphorylated, annealed, and ligated into Xhol and Xbal-cut vector plasmid pBSK to obtain the pBSK-URE plasmid. The 160-bp, AU-rich subfragment of the p21 WAF1/CIP1 3' UTR present in plasmid CMV-Luc-WAF clone 7.1 (19) was further subcloned in plasmid vector pBSKII (Stratagene) to obtain pBSK-Waf clone 7A.1. The 45-bp subfragment of GADD45 5' UTR, present in clone 11.1 (17), was subcloned in the pBSKII vector plasmid to obtain the pBSK-GADD clone 12.4. All of the plasmids described above are also schematically drawn in Fig. 2.

Protein encoding an open reading frame of human c-myc cDNA (36) was reverse transcription-PCR amplification using total RNA extracted from MDA-MB-468 HBC cells and a combination of sense (5'-GCGGAATTCCTTACGCACAAGAGTTCCG-3', sense primer) and antisense oligos (5'-GCGGAATTCGACGATGCCCTCAACGTT-3'; positions –5 to +15; Ref. 36) and antisense oligos (5'-GCGGAATTCCTACGCCCAAGATGGTCGG-3'; positions +1303 to +1321; Ref. 36). The EcoRI-digested PCR product was cloned in the sense orientation into the vector plasmid pcDNA3 (Invitrogen) to obtain the pcDNA3-Myc plasmid. The identity of the c-myc cDNA insert in plasmid pcDNA3-Myc was ascertained by partial sequencing using T7 and Sp-6 primers. The c-myc cDNA insert of plasmid pcDNA-Myc was subsequently used in Northern blot hybridization analysis. The plasmid pMycSD3 (24) containing ~400 nucleotides of the c-myc 3' UTR, followed by 100 nucleotides of poly(A) sequences, was kindly provided by Dr. Gary Brewer (University of Medicine and Dentistry of New Jersey, Piscataway, NJ).

Stable Transfections and Analysis of mRNA Decay. MDA-MB-468 cells were transfected independently with CMV-Luc clone 6.1, CMV-Luc-Fos 5.2, or CMV-Luc-WAF 4.2. Several neomycin-resistant (800–1000 μg/ml) sublines from each of the transfections were obtained as described before (17). The expression of luciferase, chimeric luciferase-c-fos, or chimeric luciferase-p21 WAF1/CIP1 transcripts was evaluated by Northern blot hybridization using a radiolabeled 0.6-kbp, HindIII-EcoRI luciferase cDNA fragment derived from pGL2-Basic (Promega). Two or more independent sublines containing stably integrated plasmid CMV-Luc 6.1 (19) or CMV-Luc-Fos 5.2, which showed low to moderate levels of luciferase expression, were selected for analysis of mRNA decay. OA-treated and untreated clonal derivatives of MDA-MB-468 cells were cultured in the presence of transcriptional inhibitor AMD (4 μg/ml) for various times to study the rates of decay of transfected luciferase and chimeric luciferase-c-fos transcripts, essentially per the methodology described before (17).

In Vitro Transcription and Electrophoretic Gel Mobility Shift Assay. Plasmids pBSK-Fos 10.1, pBSK-Waf 7A.1, and pBSK-GADD 12.4 were separately linearized with Xbal, and T7 promoter primer was used to synthesize unlabeled sense strand RNAs as described before (37). Plasmid pMycSD3 (24) was linearized with HindIII, and Sp-6 promoter primer was used to synthesize unlabeled sense strand c-myc 3' UTR RNA. Plasmid pBSK-URE was linearized with EcoRI, and T7 promoter primer was used to synthesize URE RNA. The labeled competitors RNAs were derived independently from plasmids pMycSD3, pBSK-Waf 7A.1, pBSK-URE, and pBSK-Fos 10.1, respectively.

Cytoplasmic protein extracts from MDA-MB-468 HBC cells were prepared essentially as described before (38). Twenty μg of cytoplasmic protein/reaction were preincubated at room temperature for 10 min with 200 ng/μl Escherichia coli tRNA and 1× binding buffer containing 12 mM HEPES (pH 7.5), 5 mM MgCl2, 1.25 mM EDTA, 1.25 mM DTT, 155 mM KCl, and 10% glycerol in a total reaction volume of 20 μl. In some cases, Proteinase K (Sigma; 5 μg/reaction), calf intestinal alkaline phosphatase (BMB; 40 units/reaction), or 1 μl of ~200 ng/μl of unlabeled sense strand RNAs was included in the preincubation step. The specific competitor RNAs consisted of either the 105-nucleotide, ARE RNA derived from plasmid pBSK-Fos 10.1 or the 159-nucleotide, ARE RNA derived from plasmid pBSK-Waf 7A.1. The nonspecific competitor RNA consisted of 45 nucleotides of 5' UTR subfragment of GADD45 mRNA derived from plasmid pBSK-GADD 12.4. Radiolabeled probe RNA (1 ng/reaction; ~10,000 cpm) was then added to the reaction mix and incubated at room temperature for an additional 30 min. The reactions were then exposed to UV and ice for 5 min (120 microjoules/min) using a UV cross-linker (Stratagene) to cross-link the proteins interacting with the RNAs. Five units of RNase T1 (BMB) and 2.5 units of RNase A (Sigma) were added to each reaction and incubated at room temperature for 30 min. Fifty μg of Heparin (Sigma) were added to each reaction, followed by incubation on ice for 10 min. The reactions were boiled and then analyzed on 12% SDS-polyacrylamide gel (ac-
rylamide-bisacylamide, 30:0.8) at 65 V in 1 X Tris glycine buffer for a period of 14–16 h. The gels were dried and exposed for autoradiography for appropriate period of times.

Acknowledgments

We are grateful to Jordan Denner and Richard Milianich of the Baltimore Veterans Affairs Medical center, Baltimore, MD, and William Browning of the John D. Dingell Veterans Affairs Medical Center, Detroit, MI, for preparing the illustrations.

References