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13. ABSTRACT *(Maximum 200 Words)*

C-CAM is an epithelial cell adhesion molecule. We have shown that loss of C-CAM is an early event in prostate cancer development and re-introduction of C-CAM into prostate cancer cells could inhibit their tumorigenic potential. These results indicate that C-CAM is a tumor suppressor. The mechanism of C-CAM down-regulation in tumorigenesis is not clear. Our hypothesis is that transcriptional down-regulation instead of deletion of C-CAM gene is the major cause. We propose to **identify mechanisms that regulate C-CAM gene expression in prostate carcinogenesis.** We have identified that AP-2 is a transcriptional activator of C-CAM and thus is a potential regulator of C-CAM expression during tumorigenesis. In addition, we have developed a novel *in vivo* functional screening method to identify new transcription factors that regulate C-CAM gene expression during prostate carcinogenesis. Preliminary study has identified several candidate genes and we are in the process of confirming their effects on C-CAM expression. Our reviewer suggested that we pursue regulation of C-CAM gene expression by steroid hormones. We have incorporated androgen regulation in our study and have made significant progress in this effort. A manuscript is under submission. Results from this study will allow us to understand C-CAM gene regulation during tumorigenesis.

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(4) INTRODUCTION

C-CAM is a tumor suppressor that is lost, by transcriptional down-regulation, early in the progression of prostate cancer. We propose to unravel the mechanism that down-regulates C-CAM gene expression in prostate carcinogenesis by identifying factors that alter C-CAM expression in malignant transformation. Specifically, we proposed to: (1) Examine whether methylation of C-CAM gene is a mechanism for C-CAM down-regulation in prostatic carcinogenesis; and (2) Characterize transcription factors that regulate C-CAM expression in prostatic carcinogenesis. The proposed work was divided into four Tasks to be carried out in parallel.

Task 1. Examine whether cytidine methylation of regulatory sequences in C-CAM gene occurs in human prostatic carcinogenesis (months 1-18)

Task 2. Examine the involvement of AP-2 in C-CAM gene expression in carcinogenesis (months 1-12)

Task 3. Search for the transcriptional activators/co-activators involved in C-CAM expression (months 1-30)

Task 4. Search for transcriptional repressors (months 1-24).

Our reviewer suggested that we pursue regulation of C-CAM gene expression by steroid hormones. We have incorporated androgen regulation in our study, and made significant progress in this effort. A manuscript is under submission, which will be briefly summarized. A second reviewer pointed out that the rationale and data on methylation as a factor seemed weak. We have performed some preliminary studies and found that methylation may not be the mechanism of transcriptional repression of C-CAM in tumorigenesis. Thus, the preliminary results (described in section 5.1) agreed with reviewers comments and Aim #1 (Task 1) was not further pursued. Data in Task 2 on the involvement of AP-2 in C-CAM gene expression in carcinogenesis will be described in section 5.2. We expect to complete this aim soon and a manuscript describing this aspect of study is in preparation. Since Task 3 will require the entire project period (months 1-30) to complete, we concentrated on this Task and have obtained a substantial amount of interesting results, as described in section 5.3 below. Task 4 has been initiated and the screening of repressor using modified yeast two-hybrid system is underway. We expect to have some results soon.

(5) BODY (Progress report)

5.1. Studies performed under Task 1--Examine whether cytidine methylation of regulatory sequences in C-CAM gene occurs in human prostatic carcinogenesis

5.1.1. Rationale: Alterations in DNA methylation are frequently observed as consistent molecular changes in human tumors (1, 2). Cytidine methylation of promoter is a common mechanism for transcriptional silencing and has been shown to be involved in the inactivation of VHL (3), p16 (4), cadherin (5), and GST-π (6), which are molecules with tumor or metastasis suppressor activities. The possibility that decreased expression of C-CAM may result from methylation of its promoter region has been examined by Rosenberg et al. (7). They found that hypomethylation of C-CAM gene was detected in some of the samples from colon carcinomas (7), suggesting possible involvement of altered methylation in the regulation of C-CAM gene expression in carcinogenesis. Whether altered methylation of C-CAM promoter is one of the mechanisms for C-CAM inactivation during prostate carcinogenesis is not clear.

5.1.2. Experimental Plan: We will determine whether differences in C-CAM promoter methylation correlate with the differences in C-CAM expression detected in the human prostatic cancer cell lines and prostate carcinoma. To test whether hypermethylation is involved in C-CAM gene inactivation, we will treat the cells with methylase inhibitor 5-deoxyazacytidine. Cells will be treated with different doses (0.3-1 uM) of 5-deoxyazacytidine for 3-5 days and C-CAM expression will be analyzed by RNase protection or western blot. Re-expression of C-CAM transcript or protein by methylase inhibitor will indicate that methylation plays a role in the loss of C-CAM expression in that cell line.

5.1.3. Results: Rat prostate cancer cells, Mat-Ly-Lu (MLL) (8), were treated with 0.3 uM or 1 uM 5-deoxyazacytidine for 3 days and C-CAM expression was analyzed by western blot using anti-C-CAM antibody. Anti-actin antibody was used as a control for protein loading. As shown in **Fig. 1**, there was no significant changes in the level of C-CAM expression in Mat-Ly-Lu (MLL) cells treated with 5-deoxyazacytidine when compared to that of control.

5.1.4. Discussion: Since treatment of prostate cancer cells with 5-deoxyazacytidine did not increase C-CAM expression, this observation suggests that methylation may not be the mechanism of transcriptional repression of C-CAM in tumorigenesis.

5.2. Studies performed under Task 2—Examine the involvement of AP-2 in C-CAM gene expression

5.2.1. Rationale: The C-CAM promoter lacks a TATA or CAAT box but has potential binding sites for known basal and regulatory transcription factors. Several AP-2 (activator protein 2) binding sites were found in the C-CAM promoter. AP-2 (activator protein 2) was shown to regulate the expression of several oncogenes and tumor suppressor genes and has been shown to involve in tumorigenesis. Thus, AP-2 is one of the known factors that may be potentially involved in C-CAM down-regulation in tumorigenesis.

5.2.2. Experimental Plan: We examined whether AP-2 has any effects on C-CAM promoter activity by co-transfecting C-CAM promoter-luciferase constructs and an AP-2 expression vector into MLL cells.

5.2.3. Results: Addition of AP-2 resulted in an 8-10-fold increase in luciferase expression when the reporter gene was driven by the -1609, -459, -249, -194 bp of C-CAM promoter (**Fig. 2**). Deletion of the C-CAM promoter down to -147 bp abolished the AP-2 effect. This result suggests that AP-2 is a transcriptional activator of C-CAM and the AP-2 responsive element is located between -194 bp and -147 bp region of the C-CAM promoter, which is consistent with prediction from promoter sequence analysis. The potential AP-2 binding site was mutated to see if it was indeed involved in AP-2 regulation. Mutations in the potential AP-2 binding site completely abolished the response (data not shown). This observation suggest that the potential AP-2 binding sequence is involved in the AP-2 regulation of C-CAM promoter activity.

Electrophoretic mobility shift assay was further used to determine whether AP-2 can bind to the promoter sequence. A double-stranded oligonucleotide containing the promoter sequence between -194 to -147 was used in an electrophoretic mobility shift assay. **Fig. 3B** showed that nuclear extract prepared from MLL cells can bind to the (-194 to -147) oligonucleotide and the binding can be specifically competed by the unlabeled corresponding oligonucleotide duplexes .

However, the binding can not be supershift by antibody against AP-2. On the other hand, nuclear extract prepared from MLL cells transfected with AP-2 expression vector showed binding to the AP-2 consensus sequence and the binding can be supershifted by antibody against AP-2 (**Fig. 3A**). These observations suggest that AP-2 does not bind to the C-CAM promoter directly and it may regulate C-CAM promoter activity through modulation of other transcription factors. Because the potential AP-2 binding site present in C-CAM promoter is similar to SP-1 binding site and AP-2 has been shown to interact with SP-1, we are in the process of investigating the possibility that AP-2 may modulate C-CAM promoter activity through SP-1.

5.2.4. Discussion: These results suggest that AP-2 is a transcriptional activator of C-CAM and thus is a potential regulator of C-CAM expression during tumorigenesis. To further test this notion, we will study whether AP-2 expression is altered in prostate cancer cells by Northern blot analysis. If AP-2 shows a similar pattern of down-regulation as that of C-CAM, it is very likely that it plays a role in the reduced C-CAM expression during carcinogenesis. To assess the ability of AP-2 to activate endogenous C-CAM gene expression, AP-2 will be transfected into prostate cancer cells. The functional consequence of AP-2 expression will be examined by testing whether these AP-2 expressing prostate cancer cells have lower *in vivo* tumorigenicity in nude mice.

5.3. Studies performed under Task 3— Search for the transcriptional activators/co-activators involved in C-CAM expression

5.3.1 Rationale: We hypothesized that decreased C-CAM transcription during tumorigenesis is due to a loss of activator(s) or coactivator(s). Such a factor should have the following properties: It should (1) exhibit different levels of expression between normal and cancer cells, (2) be able to activate C-CAM expression, and (3) suppress tumorigenicity of prostate cancer cells. We will identify the potential tumor-specific transcription factors based on these functional criteria.

5.3.2. Experimental plan: We plan to functionally identify these factors in the context of the native C-CAM promoter, using a tumor cell line with a low level of C-CAM expression such that modulation of C-CAM expression can be easily detected. Since C-CAM is a membrane protein, cells that have increased C-CAM expression can be selected by C-CAM antibody binding

followed with fluorescence activated cell sorting (FACS). The outline of experimental plan is shown in **Fig. 4**. The Dunning rat prostate cancer cell lines have the properties suitable for our purpose. Dunning 3327 prostate cell line was isolated from rat prostate tumor by Dunning (9) from inbred Copenhagen male rat. Sublines with different biological characteristics are available (8), which represent tumors ranging from relatively benign, slow growing, differentiated, and androgen-sensitive tumors to rapidly growing, anaplastic (AT-2), and hormone-insensitive malignant tumors (AT-3.1 and Mat-Ly-Lu). We first characterized C-CAM expression in these cell lines to determine if C-CAM protein expression levels show distinct tumor-specific down-regulation.

5.3.3. Results

5.3.3.1 Western immunoblot analysis of C-CAM protein expression in the Dunning series prostate cancer cell lines: As shown in **Fig. 5**, distinct changes in C-CAM protein expression in the Dunning cell lines are observed by western blot analysis. A significant decrease in C-CAM protein levels occurred at the transition from normal cells (NbE) to carcinoma, AT-2 and AT-3.1, followed with further reduction in rapidly growing tumors, Mat-Ly-Lu (MLL). A normal prostate cell line NbE, derived from ventral prostate of Noble rat (10), was used as a control. In Mat-Ly-Lu cell line, C-CAM expression level is about 4% as compared to that of the NbE control cell line. MLL cell line was selected for further studies.

5.3.3.2 Transfection of human prostate cDNA library in mammalian expression vector into Mat-Ly-Lu and FACS analysis: To screen for C-CAM activation factors, a human prostate cDNA library in a mammalian expression vector was constructed and used to transfect MLL cells. Three million MLL cells were transfected with 15 ug of expression vector using lipofectamine (Gibco/BRL). At 2 days post-transfection, these cells were trypsinized from the plates and incubated with polyclonal anti-C-CAM antibodies followed with FITC-conjugated secondary antibody. The top 2% fluorescence positive cells, which were considered C-CAM positive, were separated from total cell populations by FACS.

5.3.3.3 Isolation of plasmid DNA from C-CAM positive cells: Total DNA was isolated from the FACS sorted cells and electroporated into E. coli, which were selected in ampicillin-

containing agar plates (library plasmids contain β-lactamase). Ampicillin-resistance colonies were recovered and pooled, and their plasmid DNA (first round DNA) (15 ug) were used to transfect seven million MLL cells. The top 4.5% C-CAM positive cells were isolated by FACS, and these plasmid DNAs (second round DNA) were retrieved as above. This selection procedure was repeated two more times and the resulting DNAs were analyzed by restriction enzyme digestion.

5.3.3.4 Results from 4 cycles of FACS selection: As shown in **Fig. 6**, the original prostate cDNA library contained DNA inserts with various sizes and appeared as a smear (lane 2). Enrichment of certain insert sizes was apparent following four-cycle selection (lane 6). Plasmids from the fourth selection were transformed into bacteria and plasmids from single bacteria colonies were isolated and sequence determination is underway. One of the plasmids contains a gene encoding kid-1, a zinc-finger protein, consistent with the involvement of transcription factors in C-CAM regulation. These plasmids will be transfected into MLL to confirm their ability to activate C-CAM gene promoter. Plasmids that consistently activate C-CAM expression in MLL cells will be further analyzed.

5.3.4 Discussion: The concept of functional screening of molecules that modulate C-CAM expression in tumor cells was considered to be novel, but risky, by the reviewers. With the funding, we were able to evaluate the method and improve the efficiency and accuracy of the steps taken. Our modifications include (1) Use of the rat MLL cells instead of human prostate cancer cell lines. The reason is that the C-CAM antibodies react with rat C-CAM well but only react weakly with human C-CAM. This cell line also allows us to study C-CAM gene regulation in its natural context. (2) We have compared several transfection methods/reagents and found that lipofectamine can produce about 30% transfection efficiency in MLL cells. (3) Electroporation of isolated plasmids into competent E. coli greatly increases the efficiency of transformation. This step not only amplifies selected plasmids, but also removes contaminant mitochondria or cellular DNA. With these improvements, we are confident that factors that modulate C-CAM expression can be isolated.

5.4. Studies on androgen regulation of C-CAM gene expression--(manuscript attached)

5.4.1. Rationale: In our previous studies using castration-induced prostate involution together with administration of androgen or antiandrogen, we found that the expression of C-CAM in rat ventral prostatic epithelia was regulated by androgens (11, 12). However, it is not

clear whether this androgen regulation of C-CAM expression is direct or indirect. Therefore, we investigated whether androgen receptor (AR) can directly regulate the C-CAM promoter.

5.4.2. Experimental Plan: The effect of androgen and AR on C-CAM expression was studied by co-transfecting a reporter plasmid containing the C-CAM promoter and an expression plasmid containing AR. Deletion analysis was performed to locate the androgen response element in C-CAM promoter.

5.4.3. Results

5.4.3.1. Localization of androgen-responsive region in the C-CAM promoter: A series of C-CAM promoters with progressive 5' deletions were cloned in front of the luciferase reporter and cotransfected with an AR expression vector, pAR, into HeLa cells. Upon R1881 stimulation, AR can enhance C-CAM promoter activity in a ligand-dependent manner, and the AR response element (ARE) resides within a relatively short (-249 to -194) region in the 5' flanking region of the C-CAM promoter (see manuscript attached).

5.4.3.2. Test the effect of mutant AR on C-CAM promoter activity: AR is a 100 kDa protein containing a DNA binding domain in its N-terminal region and a transcription activation domain in its C-terminal region. To test whether activation of the C-CAM promoter by androgen is due to direct interaction between AR and the C-CAM promoter, we examined the effect of a mutant AR (AR64), which has a mutated (defective) DNA-binding domain, on C-CAM promoter activity. In contrast to the wild-type AR, this mutant AR (AR64) did not mediate any hormone inducible stimulation at the C-CAM promoter (data not shown). This result suggests that activation of C-CAM promoter by wild-type AR is likely through direct AR-promoter interaction.

5.4.3.3. Identification of AR-interacting sites in C-CAM promoter: Two potential AREs (ARE-1 and ARE-2) were found between -249 and -197 bp in the C-CAM promoter. These two potential ARE sites were mutated to see if they mediate androgen regulation. Only mutation of the second site abolished the androgen-stimulated response (data not shown). These results suggest that the sequence between -248 and -243 (ARE-2) is critical for androgen regulation of C-CAM promoter.

5.4.4. Discussion: We show that AR regulates C-CAM transcription in a ligand-dependent manner and this regulation requires sequences within the -249 C-CAM promoter. In addition, AR, through its DNA binding domain, directly interacts with the C-CAM promoter, suggesting

that AR regulation of C-CAM expression is, at least in part, mediated by a direct mechanism. This study also establishes that androgen receptor is one of the transcriptional regulators of C-CAM gene expression.

(6) KEY RESEARCH ACCOMPLISHMENTS

- Confirm that methylation of C-CAM promoter is not the major mechanism of C-CAM regulation in prostate tumorigenesis.
- Identify that AP-2 is one of the transcriptional regulators of C-CAM expression
- Identify several candidate genes that may have effects on C-CAM expression
- Describe the role of androgen/androgen receptor in regulating C-CAM gene expression

(7) REPORTABLE OUTCOMES

1 manuscript submitted for publication: Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.: Transcriptional regulation of C-CAM1 gene by androgen receptor.

1 abstract presented at 10th International Workshop of CEA family genes (Sept. 2-5, 1999) Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.: Transcriptional regulation of C-CAM1 gene by androgen receptor.

(8) CONCLUSION

We propose to elucidate the mechanisms that regulate C-CAM gene expression in prostate carcinogenesis. We have identified at least two transcription factors, AP-2 and the androgen receptor, that are involved in the up-regulation of C-CAM gene expression. In addition, we have developed a novel *in vivo* functional screening method to identify new transcription factors that regulate C-CAM gene expression during prostate carcinogenesis. Results from this study will allow us to better understand the regulation of C-CAM gene during tumorigenesis and this may lead to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

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Fig. 1. Effect of 5-deoxyazacytidine on C-CAM expression. Mat-Ly-Lu (MLL) cells (1 x 10^6 cells) were treated with (+) or without (-) 1 uM of 5-deoxyazacytidine for 3 days. The cells were lysed in SDS sample buffer and analyzed by western immunoblot using antibody against C-CAM. NbE cells, which is a normal prostate epithelial cell line derived from Noble rat, was used as a positive control.

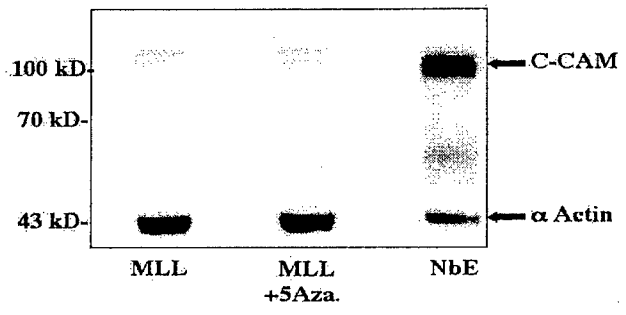


Fig. 2. Regulation of the C-CAM expression by AP-2. A series of reporter plasmids containing C-CAM promoter fragments with different 5' deletions were co-transfected with mammalian expression vector containing the AP-2 gene into MLL cells. Luciferase activities of these cell lysates were determined and reported as averages ± S.D. in relative light units from triplicate transfections

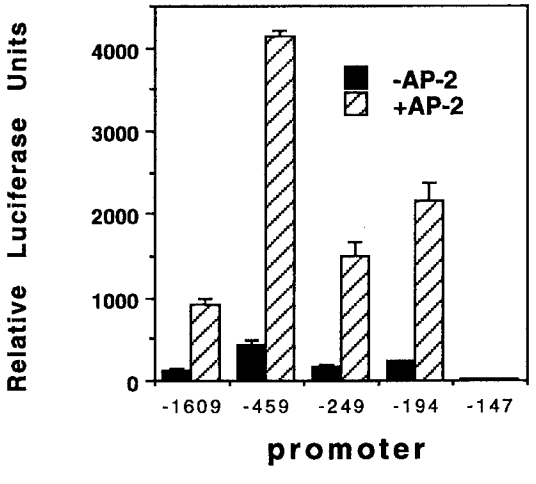


Fig. 3. Electro-mobility gel shift assay. EMSA was carried out with nuclear extract prepared from MLL cells without or with transfection with AP-2 or Luciferase expression vector using (A) the labeled double strand oligonucleotide probe containing AP-2 consensus sequence as the probe or (B) the labeled double strand oligonucleotide probe containing sequence -194 to -147 of C-CAM promoter.

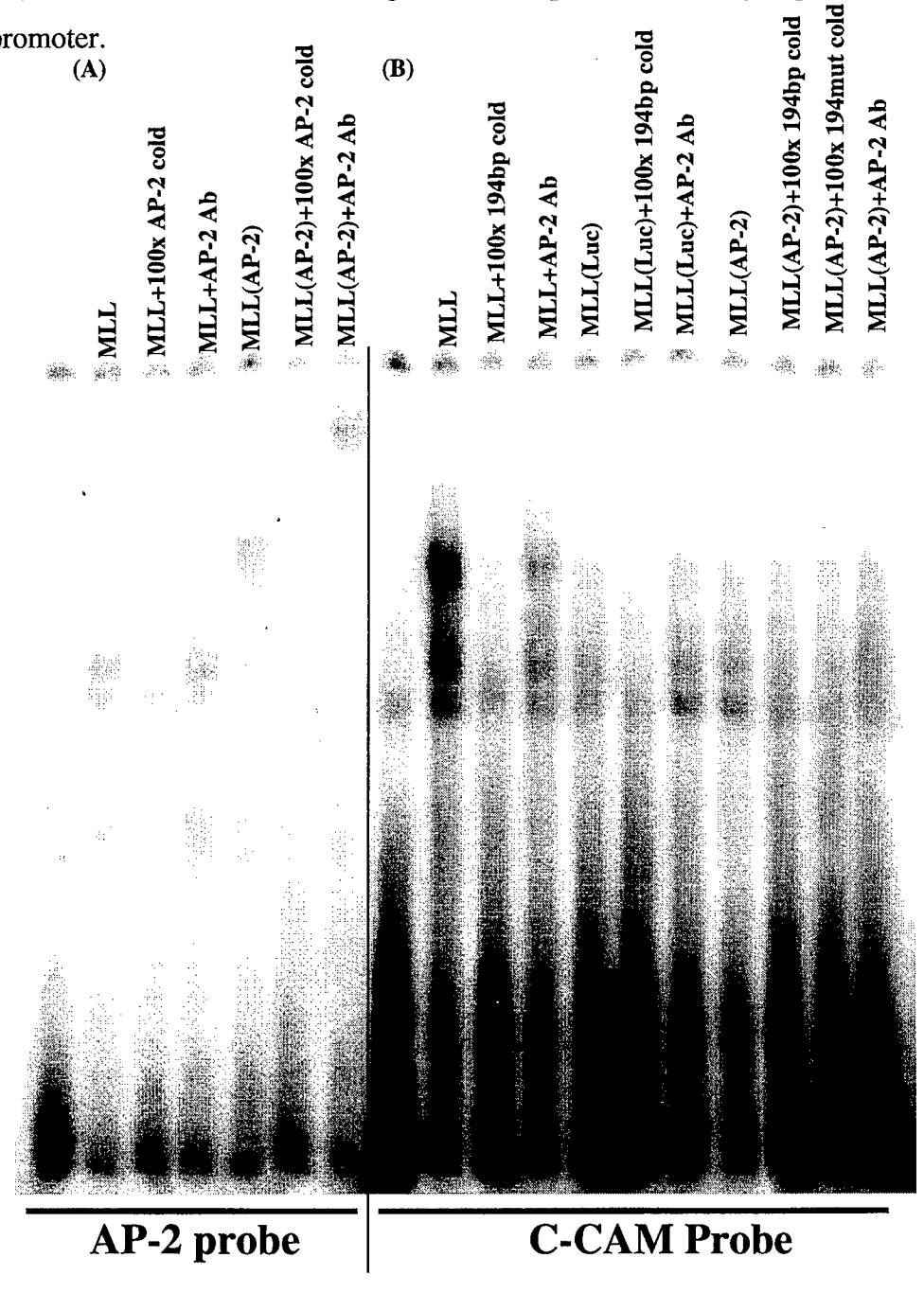


Fig. 4. Functional screening of molecules that activate C-CAM expression.

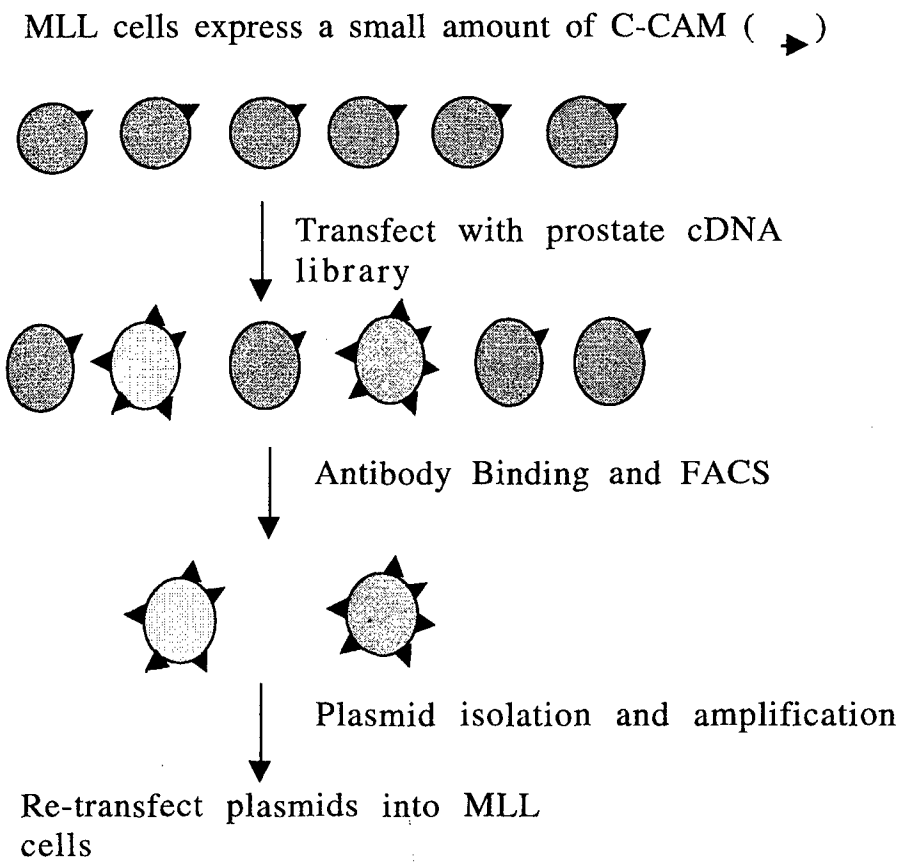


Fig. 5. Western immunoblot analysis of C-CAM expression in Dunning rat prostate cancer cell lines. The levels of C-CAM protein expression in Dunning rat prostate cancer cell lines (AT2, AT3.1, and Mat-Ly-Lu (MLL)) were examined by western immunoblot analysis using antibodies against C-CAM. A normal prostate cell line NbE, derived from ventral prostate of Noble rat, was used as a control. A significant decrease in C-CAM protein levels occurred at the transition from normal to carcinoma (i.e., AT2, and AT3.1), followed with further reduction in C-CAM protein level in the metastatic subline MLL. In the MLL cell line, the C-CAM expression level is about 4%, compared with that of normal control cells.

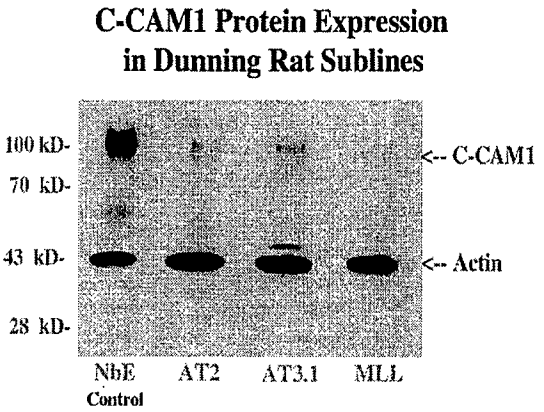
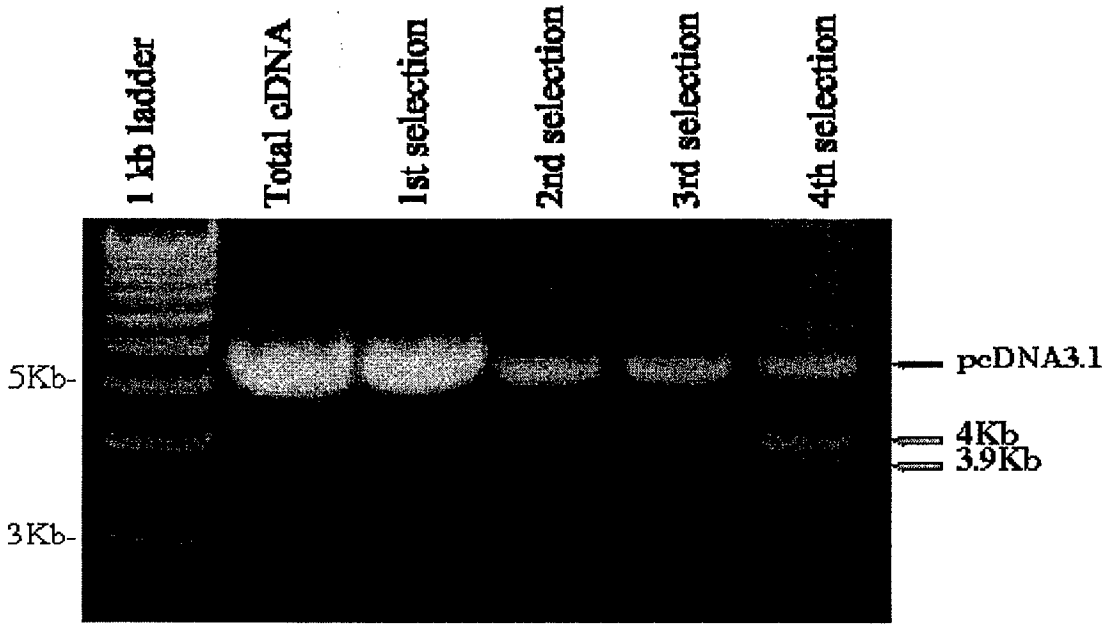


Fig. 6. Restriction digest profile of the plasmids isolated from cells selected from FACS. The original prostate cDNA library contained DNA inserts of various sizes and appeared as a smear (total DNA). Enrichment of certain insert sizes was apparent following four-cycle selection.



Androgen Regulation of Cell Adhesion Molecule (C-CAM) Gene

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Running Title: Androgen regulation of C-CAM1 gene transcription

Keywords: C-CAM1, cell adhesion molecule, androgen receptor, tumor suppressor, prostate.

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Abbreviations used: C-CAM, cell-cell adhesion molecule; CEA, carcinoembryonic antigen; Ig, immunoglobulin; BGP, biliary glycoprotein; PCR, polymerase chain reaction.

The nucleotide sequences used in this paper are in the GenBank/EMBL Data Bank with accession numbers U27207 for 5'-flanking region of rat C-CAM/pp120 gene.

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Abstract

Previous studies have established that C-CAM1 cell adhesion molecule functions as a tumor suppressor in prostate cancer and C-CAM1 is involved in the regulation of prostate growth and differentiation. However, the molecular mechanism that modulates C-CAM1 expression in prostate is not well defined. Since growth of prostate epithelial cells is androgen-regulated, we investigated the effects of androgen and androgen receptor (AR) on C-CAM1 expression. Transient transfection experiments showed that AR can enhance the C-CAM1 promoter activity in a ligand-dependent manner and that the regulatory element resides within a relatively short (-249 to -194) stretch in the 5' flanking region of the C-CAM1 gene. This androgen-regulation is likely through direct AR-promoter binding since mutant AR defective in DNA binding failed to up-regulate reporter gene expression. Furthermore, electrophoretic mobility shift assay demonstrated that the androgen receptor specifically binds to this sequence. Mutation analysis of the potential ARE sequences revealed a region within this sequence that was required for AR to activate C-CAM1 gene. The regulation of C-CAM1 gene expression by androgen could be one of the mechanisms by which androgen regulates prostatic function.

INTRODUCTION

C-CAM1 is a cell adhesion molecule of the immunoglobulin supergene family (Lin and Guidotti, 1989, Lin et al., 1991). C-CAM1 is mainly expressed in epithelial cells of many different tissues including the prostate (Odin, et al., 1988). Loss of C-CAM1 is an early event in prostate cancer progression (Kleinerman, et al., 1995, Pu, et al., 1999), suggesting that C-CAM1 may play an important role in prostate tumorigenesis. Consistent with this hypothesis, expression of C-CAM1 in prostate cancer cells can suppress their tumorigenicity *in vivo* (Hsieh, et al., 1995, Luo, et al., 1999, Estrera, et al., 1999). These observations suggest that C-CAM1 function as tumor suppressor in prostate cancer.

The prostate is an androgen-dependent organ; androgen is the major regulator of prostate development, growth and secretory function. Induction of prostate involution by androgen ablation is one of the most effective treatments for late stage prostate cancer. Since C-CAM1 is a tumor suppressor of prostate cancer, it is important to know whether expression of C-CAM1 in prostate is regulated by androgen.

The rat C-CAM1 promoter belongs to the GC-rich class of TATA-less promoters (Najjar, 1996). Deletions and substitution analyses revealed that the three proximal Sp1 binding sites are essential for basal transcription of the C-CAM1 gene. In addition, Najjar et al. (Najjar, 1996) have shown that C-CAM1 promoter activity was stimulated 2-3 fold by insulin, dexamethasone, and cAMP treatment. However, the effect of androgen on C-CAM1 promoter activity had not been examined. In this study, we examined whether the AR regulates C-CAM1 promoter activity.

MATERIALS AND METHODS

Plasmid constructions

The 5'-flanking region of rat C-CAM1 gene was cloned as previously described (Najjar, 1996). Nucleotides are numbered relative to +1 at the ATG translation initiation codon and labeled as negative numbers to reflect their position as upstream (5') of the ATG site. Using PCR, 5' deletion products (-1609bp, -439bp, -249bp, -194bp, -147bp, -131bp, -124bp, -112bp) of the C-CAM1 gene were synthesized and subcloned at the XhoI and HindIII sites of pGL3-BASIC plasmid (Najjar, 1996).

Mutants -249pLucARE-1Mut and -249pLucARE-2Mut were generated by site-directed mutagenesis of the p-249Luc vector using PCR. Oligo #305 (reverse primer) (AAGCTTTTCTCTTGGGGAAGA) and oligo #306 (forward primer) (*CTCGAG*ATGTTCTAGAACAATGAACCGAAAAGAGATCCCGCGAAGGATGGGAGGACAGC A) were used as primers to introduce substitutions in the ARE-1 region, while oligo #307 (forward primer) (GCTAGCCCGGG*CTCGAG*AGTCGACAGAACAATGAACCGAAAA) and #306 were used to introduce substitutions in the ARE-2 region. The sequences that were changed from the wild type were underlined. After these PCR products were sequenced to confirm the mutations, they were subcloned at the XhoI and HindIII sites of pGL3- BASIC plasmid (Promega, Madison, WI). The construction of the reporter plasmid harboring two androgen response elements and a TATA-box driving the luciferase gene (p[ARE]2-E1b-luc) have been described previously (Jenster, et al., 1997).

The human AR cDNA expression vector (pAR_0) was constructed with the SV40 early promoter and the rabbit ß-globin poly-adenylation signal as previously described (Brinkmann, et al., 1989). The AR mutant expression vector pAR64, with first zinc finger in the AR disrupted by the replacement of two cysteines with serine and phenylalanine, was constructed as described in Jenster et al. (Jenster, et al., 1993). The super-AR expression vector pcDNA-AR_0p65 was constructed by inserting the Asp718 (filled in with Klenow) and SacII digested fragment of pcDNA-AR_{LBD}-p65 (Sui, et al., 1999) into the HpaI and SacII digested pcDNA-AR_0mcs vector (Sui, et al., 1999). This resulted in generation of fusion protein containing wild type AR fused with the transactivation domain of p65/RelA.

Cell Culture and Transfection of HeLa cells

HeLa cells (human epithelial cervix carcinoma) (American Type Culture Collection) were maintained in minimal essential medium supplemented with 10% fetal calf serum. These cells (50,000) were plated in a 12-well plate with 10% charcoal-stripped fetal calf serum 24h before transfection. Cells were transfected with 0.3 ug of luciferase reporter plasmid containing C-CAM promoter fragment, and 0.3 ug of receptor plasmid containing either wild type (pAR_0) or modified androgen receptor (pAR64 or pcDNA-AR_0p65) per well using Lipofectin (Life Technologies, Inc., Grand Island, New York) according to the manufacturer's guidelines. Twenty four hours post-transfection, cells were washed and fed with medium containing stripped serum with or without R1881 (17α-methyltrienolone) (NEN Life Science Products, Boston, MA) and the incubation were continued for an additional 24 h. Cells were lysed in 200 ul lysis buffer and luciferase activity was measured using a luciferase assay system (Promega, Madison, Wisconsin). Experiments were performed in triplicate.

Electromobility Shift Assay

Electromobility Shift Assay (EMSA) was carried out as described in a bandshift assay system (Promega). The plasmid pRSET-GST-AR_{DBD} containing sequence from AR DNA binding domain fused to GST was constructed by inserting the Klenow treated RsrII/XbaI 0.3 kb of ARDBD fragment from AR126 (Jenster, et al., 1995) into the Klenow treated NcoI/HindIII digested pRSET-GST-SRC782-1139 vector (Spencer, et al., 1997). The GST-fusion protein containing androgen receptor DNA binding domain (GST-AR_{DBD}) was expressed and purified from E. coli BL21(DE3) and used for EMSA.

RESULTS

Localization of an androgen-responsive region in the C-CAM1 promoter

C-CAM1 promoters with different lengths, constructed by 5' deletion, were cloned in front of the luciferase gene in the reporter plasmid. Each of these plasmids were transiently co-transfected with the androgen receptor expression vector pAR_0 into HeLa cells (Brinkmann, et al., 1989). The reporter plasmid containing two androgen response elements and a TATA-box derived from E1b gene (p[ARE]2-E1b-luc) was used as a positive control. In the absence of the androgen analogue R1881, the 1609 basepair (bp) C-CAM1 promoter mediated a 106-fold increase in reporter gene expression as compared to the reverse oriented C-CAM1 promoter fragment (Fig. 1). Deletion of the C-CAM1 promoter up to bp -194 did not abolish its ability to induce luciferase expression, while deletion up to bp -147 markedly reduced the promoter activity (Fig. 1). This result suggests that a minimal promoter is located within the first 194 bp 5' from C-CAM1's translation start site. We next investigated whether androgen has an effect on C-CAM1 promoter. As shown in Figure 1, the plasmid containing the C-CAM1 promoter region from bp –249 to bp -21 exhibited a 2.5-fold increase in luciferase activity upon addition of androgen analogue R1881. In contrast, no significant hormone response was observed with plasmids containing the entire 1609 bp, 439 bp, or the 194 bp segment proximal to the translation start site. These observations suggest that region between bp -249 and -194 in C-CAM1 gene may contain an androgen-regulated sequence.

Direct binding of AR to the promoter sequence

The AR is a 110-112 kDa protein containing transcriptional activation domains in its N-terminal region, a centrally located DNA binding domain, and the ligand binding domain at its C-terminus (Jenster, et al., 1995, Spencer, et al., 1997, Dai and Burnstein, 1996, Jenster, 1999). To test whether activation of C-CAM1 promoter by androgen is due to direct interaction between AR and C-CAM1 promoter, we investigated the effect of a mutant AR (AR64), which is defective in DNA binding (Jenster, et al., 1993), on C-CAM1 promoter activity. In contrast to the wild-type AR, the mutant AR (AR64), when co-transfected with -249pLuc into HeLa cells, did not show any detectable hormone induction (Fig. 2). Similarly, p[ARE]2-E1b-Luc also lost its response to R1881 stimulation

(Fig. 2). This result suggests that activation of the C-CAM promoter by the wild-type AR requires its DNA binding domain and thus, AR may bind to C-CAM1 directly.

Electrophoretic mobility shift assay was further used to determine whether AR can bind to the promoter sequence. A double-stranded oligonucleotide containing the promoter sequence between -249 to -194 was used in an electrophoretic mobility shift assay. Fig. 3 showed that AR DNA binding domain can bind to the (-249 to -194) oligonucleotide and the binding can be specifically competed by the unlabeled corresponding oligonucleotide duplexes and also by an unlabeled oligonucleotide containing AR consensus sequence (Roche, et al., 1992). This observation suggests that AR binds specifically to the C-CAM1 promoter sequence.

Identification of AR-interacting sites

Using a DNA-binding site selection assay, Roche et al. (Roche, et al., 1992) have determined a consensus AR DNA-binding site for AR. Two regions in the promoter, located at bp –215 to -220 and –243 to –248, respectively, in the C-CAM1 promoter show homology to the consensus sequence and could be responsible for androgen-induction of the -249pLuc reporter activity (Fig. 4). These two potential androgen receptor binding sites (ARE-1 and ARE-2) were mutated to see if they are indeed involved in androgen regulation. The effect of mutations on ARE-1 or ARE-2 on the promoter activity was examined. Mutations in ARE-1 did not cause significant change of C-CAM1 promoter's response to R1881 while mutations in ARE-2 sites completely abolished the response (Fig. 4). In addition, mutations of both ARE-1 and ARE-2 sites have similar effect as ARE-2 mutation. These observations suggest that only ARE-2 site is involved in the AR regulation of C-CAM1 promoter activity.

The mutational analysis of potential ARE sites was also examined with super-AR, containing AR fused with the transactivation domain of p65/RelA. The AR, through its DNA binding domain, can direct p65 protein to transactivation resulting in amplification of AR-mediated transcriptional signals. As shown in Fig. 5A, the -249bp C-CAM1 promoter activity showed a 5-6-fold increase in

response to R1881 stimulation with the super-AR, in contrast to a 2-3 fold activity with the wild-type AR. Such an enhancement of reporter activity was used to further confirm the mutational analysis. In the presence of super-AR, mutation in ARE-1 showed a 4-fold increase of luciferase activity in response to R1881. Similar to those observed with wild-type AR, R1881 treatment did not increase the promoter activity of ARE-2 mutant or ARE-1&2 mutant. These observations further confirm that AR only requires ARE-2 to stimulate C-CAM1 promoter activity.

DISCUSSION

Androgen is the most important factor that regulates prostate growth and differentiation. A series of genes that have functions related to cell growth modulation were shown to be regulated by androgen in prostate. It was shown that androgen can upregulate growth factors such as EGF (Hiramatsu, et al., 1988, Nishi, et al., 1996), KGF (Peehl and Rubin, 1995, Rubin, et al., 1995, Fasciana, et al., 1996, Yan, et al., 1992), and bFGF (Katz, et al., 1989, Zuck, et al., 1992), leading to epithelial proliferation. In addition, Bcl-2, a major cell survival protein was also shown to be upregulated by androgen (Raffo, et al., 1995). Upregulation of growth hormones and pro-apoptotic factors may contribute to the growth of prostate. Although EGF expression was upregulated by androgen, EGFR, its receptor EGFR, appears to be under negative androgen regulation (Fiorelli, et al., 1991, St. Arnaud, et al., 1988). In addition, the cell-cycle regulatory proteins such as cdk2, cdk4, cyclin D3, cyclin A, p21CIP1/WAF-1, p27kip1, and p16 were also found to be regulated by androgen (Gregory, et al., 1998, Kokontis, et al., 1998, Lu, et al., 1999, Lu, et al., 1997, Knudsen, et al., 1998). These diverse androgen regulated events result in the maintenance of prostate homeostasis. Disruption of these intricately balanced androgen regulated events may lead to prostate cancer initiation and progression.

Here we showed that C-CAM1, a tumor suppressor gene, also regulated by androgen. C-CAM1 is upregulated by AR in a ligand-dependent manner when tested in vitro. This androgen regulation was controlled by only one of the two half sites of AR consensus sequence (Roche, et al.,

1992). Similar event was also observed by Dai et al. (Dai and Burnstein, 1996) in that the presence of one half site of AR consensus sequence is sufficient to upregulate the promoter of androgen receptor gene by AR. This half site interaction might not give as strong activity as the full consensus sequence as seen in probasin promoter (Kasper, et al., 1994) and PSA promoter (Zhang, et al., 1997). In addition, the longer C-CAM1 promoter, i.e. 1609 bp, did not show ligand-dependent regulation by androgen suggesting that other regulatory mechanism may present upstream of the promoter region and these may have influence on the interaction of AR with C-CAM1 proximal promoter region. Thus, the regulation of C-CAM1 expression by AR is complex and may depend on its cellular context. In this regard, it is interesting to note that in rat, C-CAM1 is mainly expressed in dorsal but not ventral prostate, although androgen receptor was expressed in both prostate lobes (Makarovskiy, et al., 1999). In addition, expression of C-CAM1 in the ventral prostate was under negative regulation by androgen in vivo while its expression in dorsal prostate showed no response to in vivo androgen manupulation (Makarovskiy, et al., 1999, Hsieh and Lin, 1994). In the mouse prostate, on the other hand, increase of C-CAM1 expression followed castration was detected on the dorsal prostate (Pu, et al., 1999). Together, these observations suggest that regulation of C-CAM1 by androgen in vivo is a complex process; although AR has positive effect on proximal region of C-CAM1 promoter, it may have negative influence on the other region of C-CAM1 promoter. AR regulation of C-CAM1 expression is then influenced by the cellular context and possibly the prostate stromal components.

Primary prostatic cancers are largely dependent on androgens for growth and survival. Most patients respond favorably to androgen ablation and antiandrogen therapy. However, virtually all patients will relapse with clinically defined androgen-independent cancer. The role of C-CAM1 in prostate cancer and in androgen-independent prostate disease was studied by examining the patterns of C-CAM1 expression during prostate cancer initiation, progression, and metastasis in the transgenic adenocarcinoma of mouse prostate (TRAMP) model, which was generated by using the rat probasin promoter to target simian virus 40 large T antigen specifically to mouse prostate (Greenberg, et al., 1995). In TRAMP mice, immunohistochemical staining using Ab669 polyclonal antibody against C-

CAM1 revealed that C-CAM1 protein was expressed in the normal prostate epithelia of TRAMP mice as well as in low-grade prostate intraepithelial neoplasia (PIN) in TRAMP mice. Expression was uniform on the luminal surfaces of these epithelia. C-CAM1 expression was noticeably reduced and the staining pattern heterogeneous in some high-grade PINs. C-CAM staining was generally absent in prostate cancer and metastatic lymph nodes. Androgen independent prostate cancer and its metastatic tumors generated in castrated TRAMP mice were also C-CAM negative (Pu, et al., 1999). Because loss of C-CAM1 expression occurred before the development of androgen-independent tumors, it is likely that the AR regulation of C-CAM1 expression is not related to loss of C-CAM during prostate cancer progression.

Other factors that have been shown to have an effect on C-CAM1 promoter include upstream stimulatory factor (USF) and hepatocyte nuclear factor-4 (HNF-4) (Hauck, et al., 1994). Shively (Chen, 1996) showed that treatment of HT-29 cells with interferon gamma (IFN-r) upregulated C-CAM1 expression. This was due to the ability of IFN-r to upregulate the expression of IRF-1 which, by binding to interferon stimulate response element (ISRE) located in C-CAM1 promoter, activated C-CAM1 transcription. Thus, the regulation of C-CAM1 expression is a combination of different transcriptional factors and AR is one of the factors.

FIGURE LEGEND

Fig. 1. Regulation of the C-CAM1 expression by androgen. A series of reporter plasmids containing C-CAM1 promoter fragments with different 5' deletions were co-transfected with wild-type androgen receptor plasmid (pAR_0) into HeLa cells. Twenty hours post-transfection, cells were incubated with (+) or without (-) 1 nM R1881. Luciferase activities of these cell lysates were determined as described in "Materials and Methods" and reported as averages ± S.D. in relative light units from triplicate transfections.

Fig. 2. Effect of AR mutation on its ability to activate C-CAM1 promoter. Cells were transfected with the -249pLuc C-CAM1 promoter together with wild-type AR (pAR_0) or mutant AR (pAR64) plasmid, respectively. Activities are presented as averages ± S.D. of triplicate transfections.

Fig. 3. Electro-mobility gel shift assay. EMSA was carried out with GST-AR_{DBD} and the labeled double strand oligonucleotide probe containing sequence from -249 to -194 of C-CAM1 promoter. Lane 1, without protein; Lane 2, with GST protein; Lane 3, with GST-AR_{DBD}; Lane 4, with GST-AR_{DBD} and a 100-fold molar excess of the unlabeled probe; Lane 5, with GST-AR_{DBD} and a 100-fold molar excess of a 27 bp oligonucleotide containing the AR consensus sequence (Roche, et al., 1992).

Fig. 4. Mutation analysis of the putative AREs in C-CAM1 promoter. Nucleotide sequence between nt -249 and -194 in rat C-CAM1 promoter is shown. Two putative androgen-responsive elements (ARE-1 and ARE-2) are underlined and the mutated sequence of the two putative androgen-responsive elements are shown. Cells were transfected with reporter plasmids containing the -249 bp promoters whose putative ARE-1 or ARE-2 sequence was mutated as described in "Materials and Methods". Activities are presented as percent of that of the corresponding plasmid containing no mutation and without R1881 treatment. Data are presented as means ± S.E. of three independent experiments.

Fig. 5. Activation of C-CAM1 promoter activity by super-AR. The C-CAM1 promoter transcription activity was examined by co-transfection of C-CAM1 promoter reporter construct (-249pLuc) and the super-AR expression plasmid (pAR_0p65) into HeLa cells. Luciferase activity was determined from cell lysates of transfected cells as described in Materials and Methods. Activities are presented as percent of that of without R1881 treatment. Data are presented as means ± S.E. of two independent experiments.

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Figure1

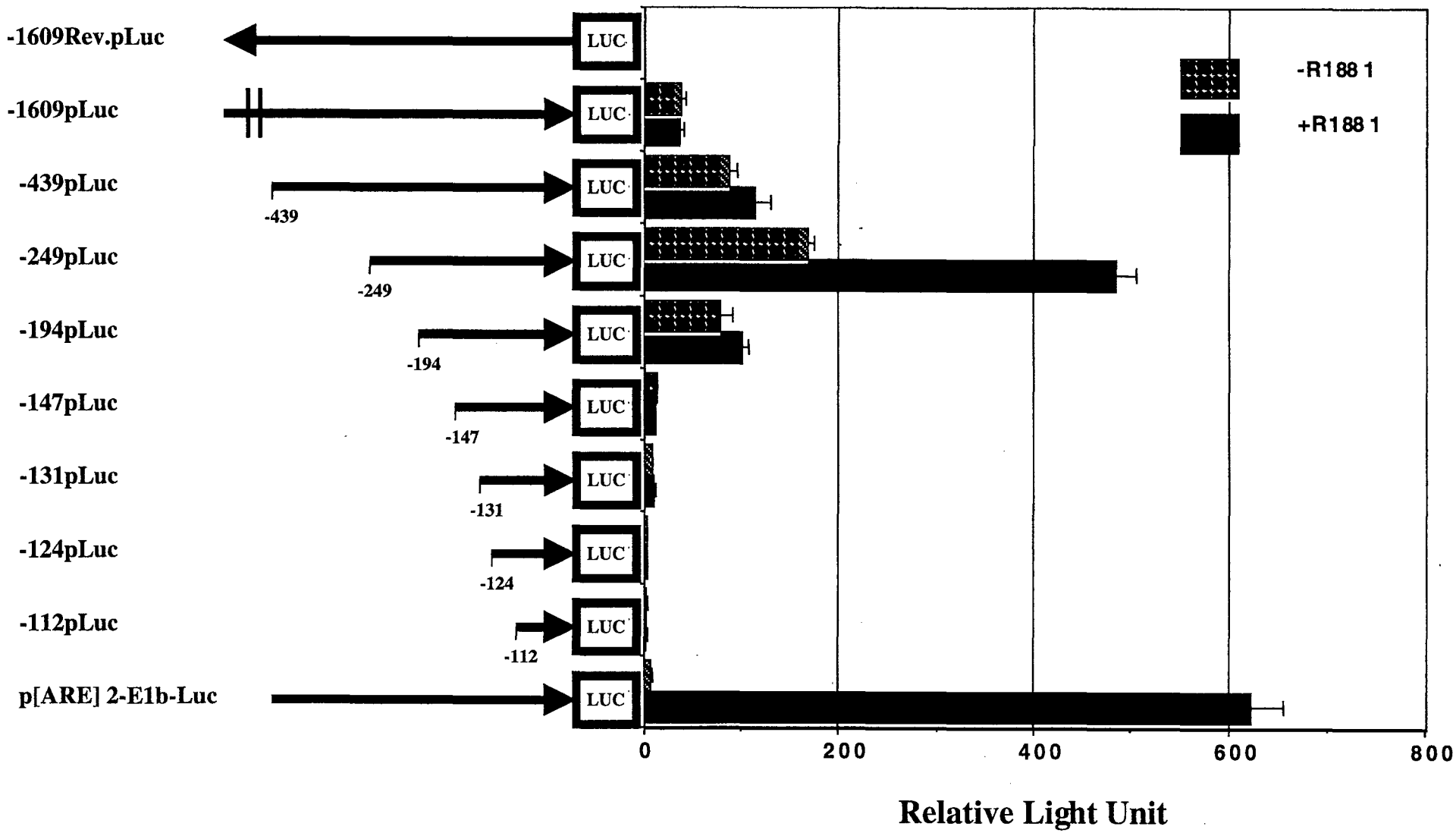

-1609Rev.pLuc
-1609pLuc
-439pLuc
-249pLuc
-194pLuc
-147pLuc
-131pLuc
-124pLuc
-112pLuc
p[ARE] 2-E1b-Luc
LUC
-439
-249
-194
-147
-131
-124
-112
-R1881
+R1881
0
200
400
600
800
Relative Light Unit

Figure 2

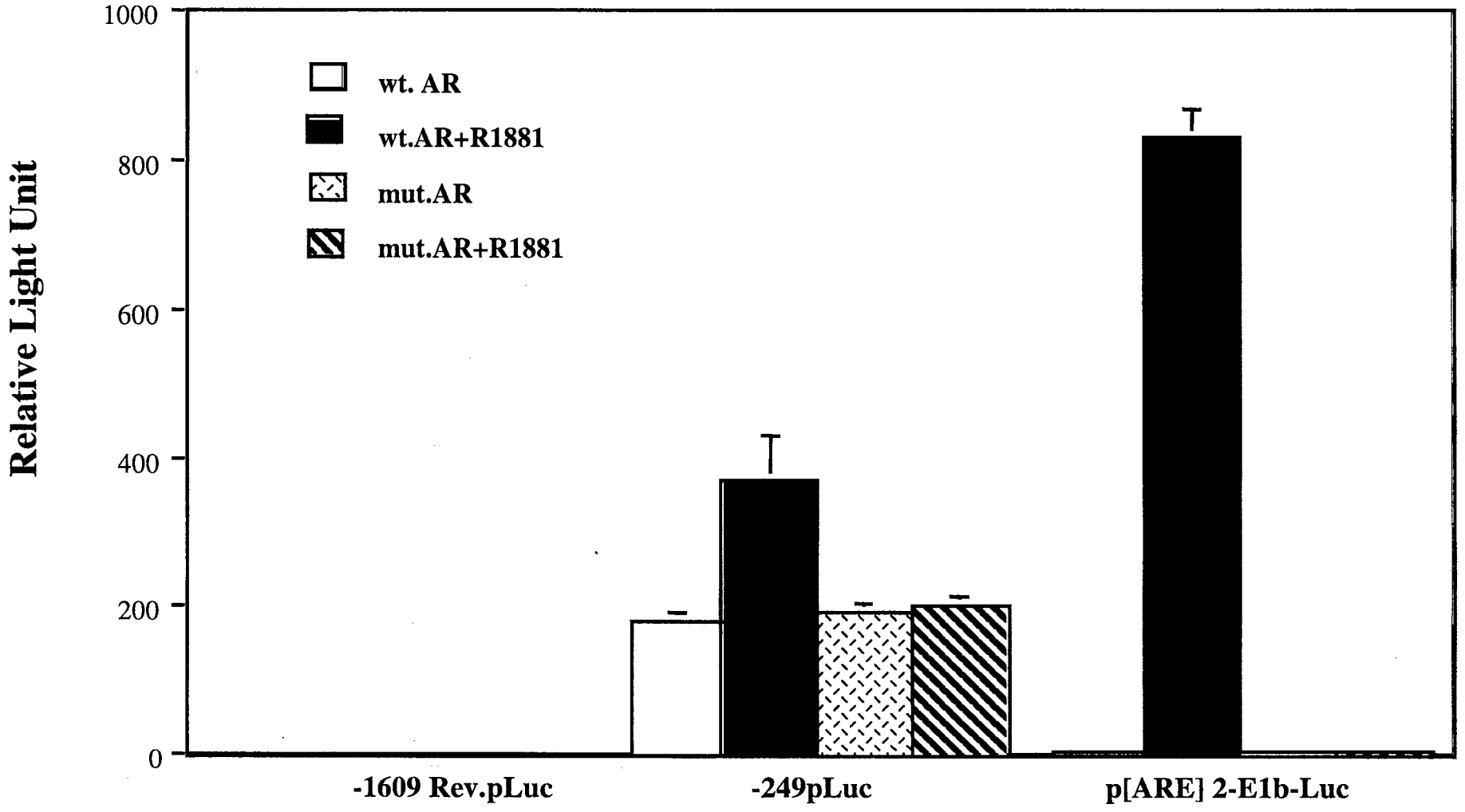

Relative Light Unit
1000
800
600
400
200
0
wt. AR
wt.AR+R1881
mut.AR
mut.AR+R1881
-1609 Rev.pLuc
-249pLuc
p[ARE] 2-E1b-Luc

Figure 3

C-CAM1 (-249 to-194)bp Probe

Lane	1	2	3	4	5
GST	-	+	-	-	-
GST-AR_{DBD}	-	-	+	+	+
50x (-249 to -194) bp Competitor	-	-	-	+	-
50x AR Consensus Competitor	-	-	-	-	+

AR-DBD ⟹

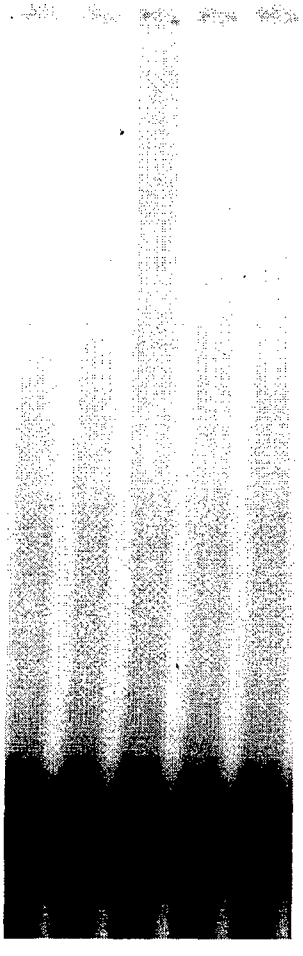


Figure 4

-249 -194

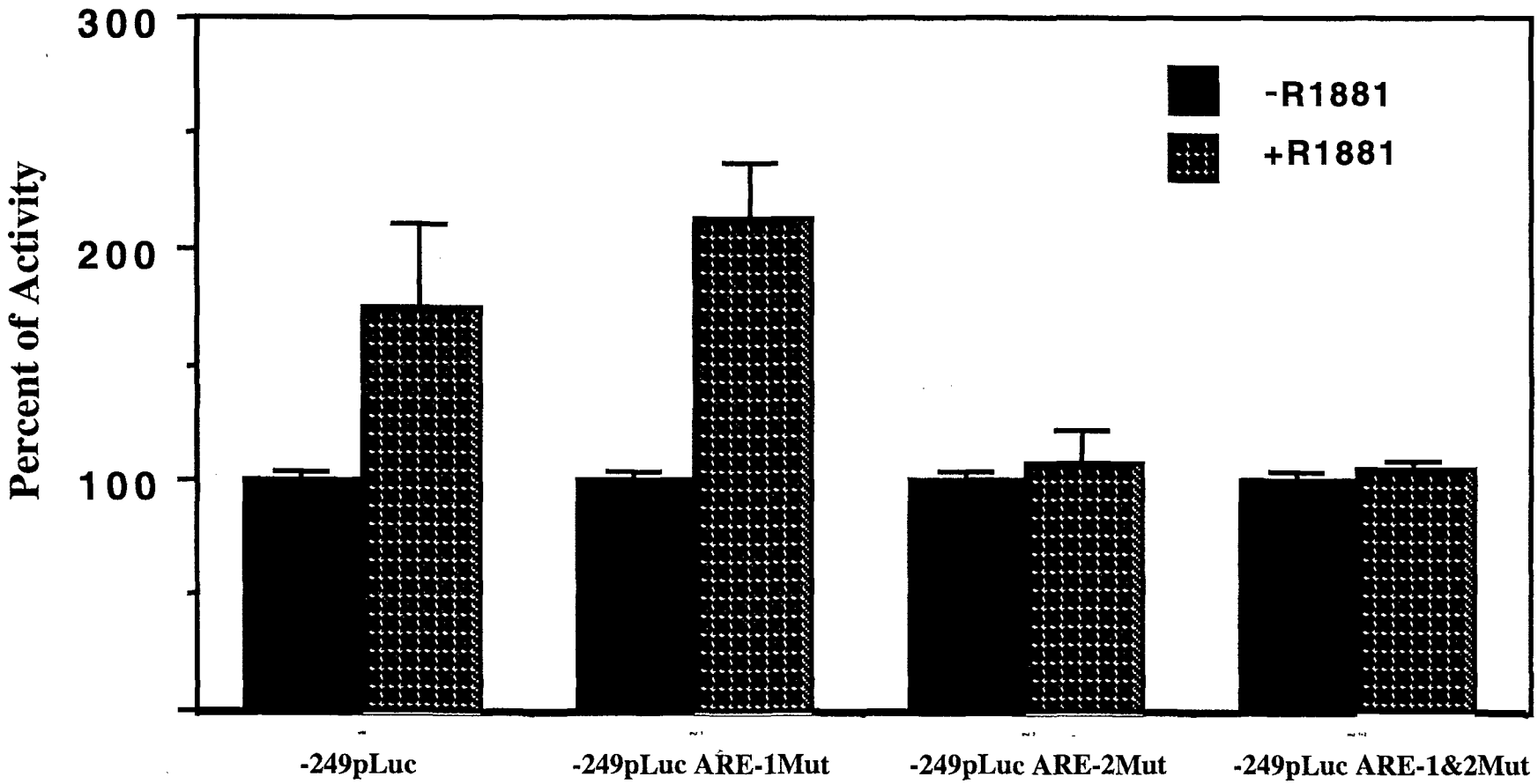
ARE-2 ARE-1

ATGTTCTAGAACAATGAACCGAAAAGAGAGGAACAGAAGGATGGGAGGACAGCACTG (-249)

.....TCCCGC..... (-249) ARE-1MUT.

.GTCGAC..... (-249) ARE-2MUT.

.GTCGAC.....TCCCGC.....(-249) ARE-1&2MUT.



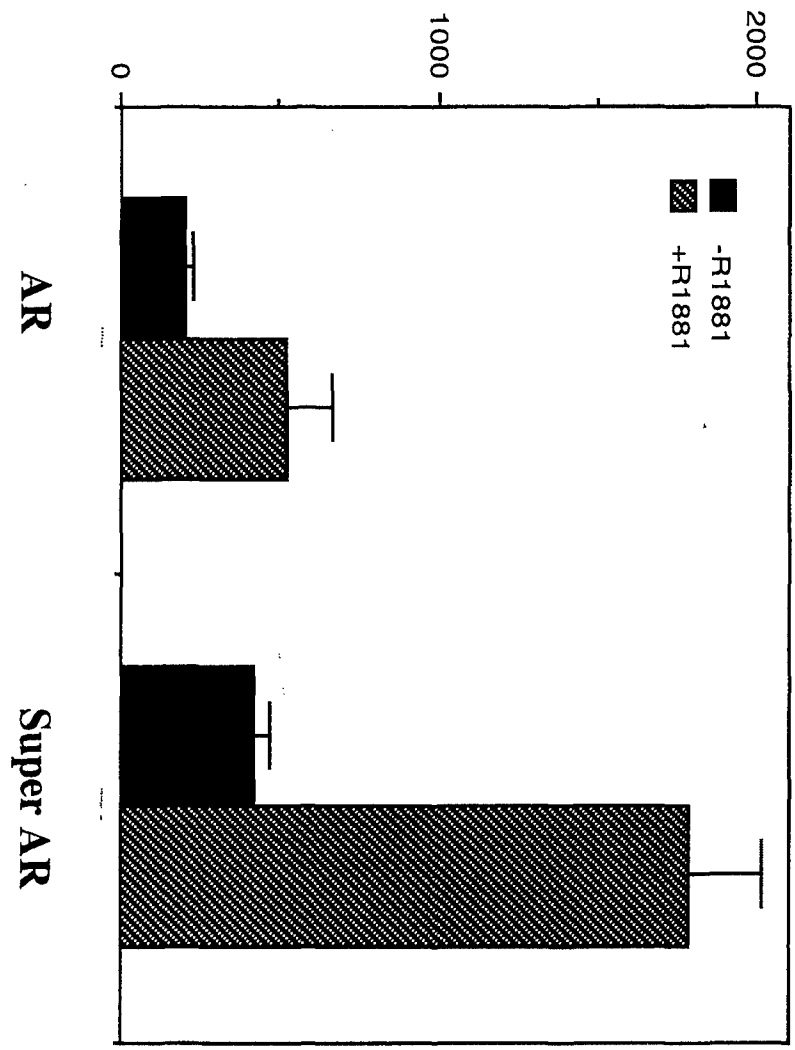
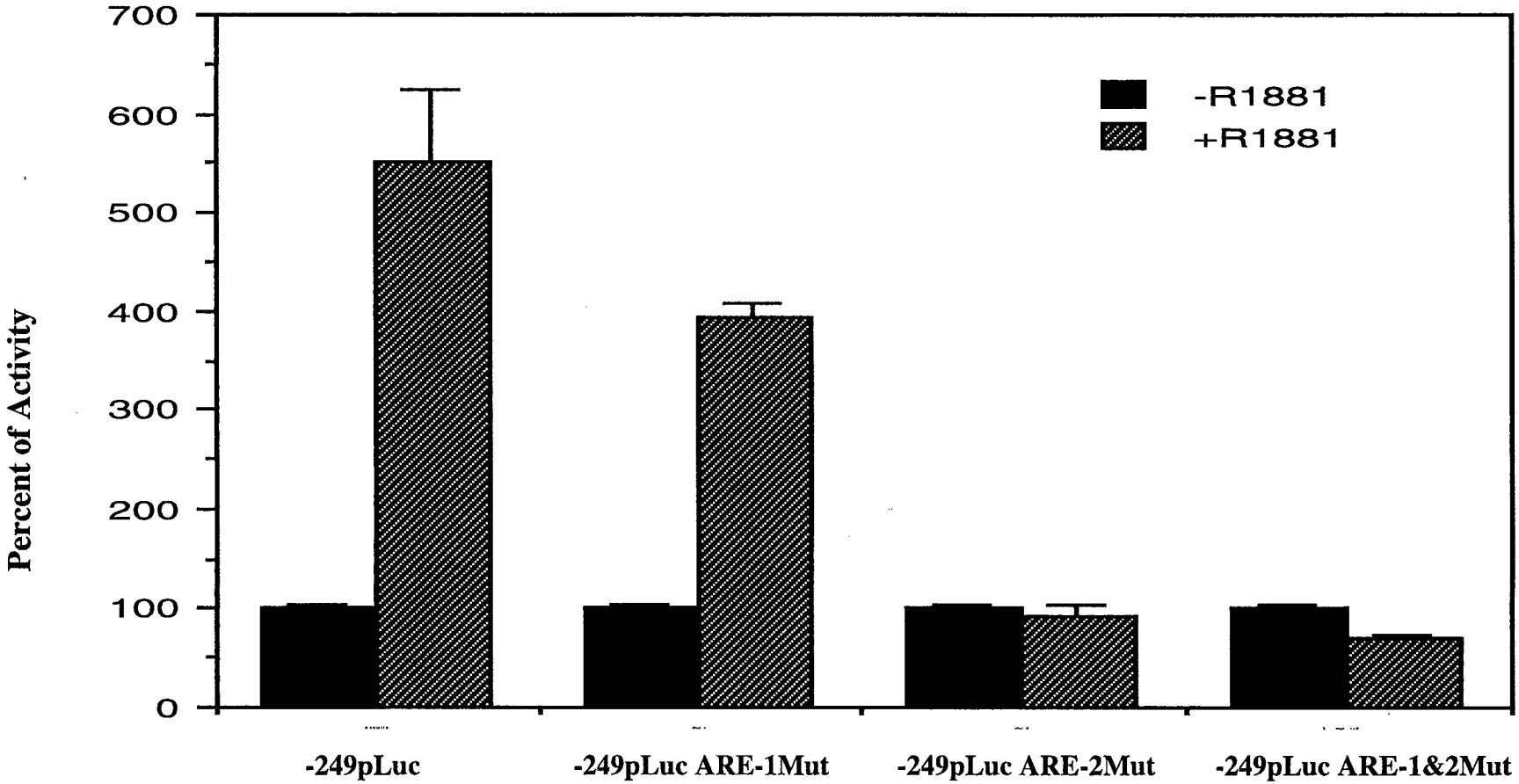


Figure 5A

Figure 5B


700
600
500
400
300
200
100
0
Percent of Activity
-R1881
+R1881
-249pLuc
-249pLuc ARE-1Mut
-249pLuc ARE-2Mut
-249pLuc ARE-1&2Mut