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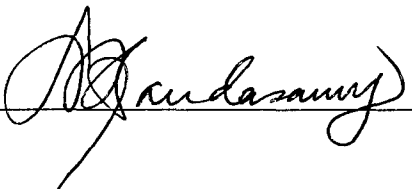
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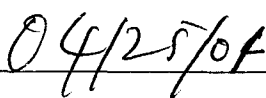
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INTRODUCTION

Breast cancer is a major cause of mortality among American women and contributes to over 50,000 deaths every year. Approximately, 1 in 11 American women contract breast cancer during their lifetime. Women with two or more first-degree relatives with breast cancer have an estimated 13-fold increased risk over the general population (1). Although 95% of breast cancers are caused by somatic mutations (sporadic breast cancers), a minority of cases (2-5%) were determined to be due to inherited mutations (familial breast cancers) (2) (Reviewed in (3)). It was also determined that families with a history of breast cancer have an inheritance pattern consistent with a highly penetrant autosomal dominant breast cancer allele, which was localized to chromosome 17 (4). These seminal studies aided in the identification and cloning of the breast cancer gene BRCA1 (5) and it has been observed that mutations in the BRCA1 gene are associated with the development of mostly high grade breast cancers (5, 6). Surprisingly, despite the presence of BRCA1 mutations in approximately 45% familial breast cancer (2), the occurrence of somatic mutations in sporadic breast cancers are rare (7). The high penetrance of inherited BRCA1 mutations, and the lack of somatic BRCA1 mutations in sporadic breast cancer, prompted proposals that loss of function/expression of BRCA1 may be mediated by epigenetic mechanisms. Consistent with this notion, several laboratories have observed CpG methylation of the BRCA1 promoter in sporadic breast cancers, as opposed to the lack of the promoter methylation in normal breast cells (8-12). This suggested that suppressed transcription of the BRCA1 gene played a role in breast cancer causation, at least in a subset of sporadic breast cancers. Furthermore, decreased expression of BRCA1 gene products were reported in high grade, invasive sporadic breast cancer and these lower levels may have occurred due to inhibition of BRCA1 transcription in a subset of cancers (13-15). Implicit in these observations is that BRCA1 may play a broader role in breast cancer causation than initially appreciated and that suppressed BRCA1 transcription may contribute to increased breast cancer risk. Some information regarding regulation of BRCA1 transcription was published (16-19). Our laboratory identified a positive regulatory region (PRR) in the BRCA1 promoter (20). Deletion of the positive regulatory region (PRR) resulted in a significant loss of BRCA1 transcriptional activity. In addition, the PRR exhibited strong and specific affinity for nuclear factors. Recently, these results were confirmed by Suen and Ross, wherein

they showed the importance of the region encompassing the PRR in the positive regulation of BRCA1 transcription (21). In the present report we present a detailed characterization of the PRR of the BRCA1 gene. We show that the PRR consists of two distinct domains, each of which possesses specific protein binding activity. Subtle deletion and point mutations in the PRR resulted in the decrease in affinities for binding proteins (data not shown) and are accompanied with concurrent decrease in the transcriptional activities of the BRCA1 mutant promoters. These results hint that the downregulation of BRCA1 expression in sporadic breast cancers could be as a result of disruption of the PRR, either by methylation or disruption of the transcription complexes assembling on the PRR. Furthermore, our results also suggest that the BRCA1 promoter positive response to the proliferative effects of estrogen in breast cells is mediated by the PRR. In addition, employing affinity purification protocols, we obtained highly enriched fractions with specific binding activity for the Py/Pu region of the PRR.

BODY

EXPERIMENTAL PROCEDURES

Mutants of the Positive Regulatory Region (PRR)- The isolation of the BRCA1 promoter, as well as the conditions used for generation of BRCA1 promoter mutants have been described previously (20). Systematic deletion and point mutants of the PRR were constructed utilizing polymerase chain reaction (PCR) amplification.

Transfections and Electrophoretic Mobility Shift Assays (EMSA)- All the experiments were performed with MCF-7 breast cancer cells. Cell cultures, transfections, luciferase assays, normalization of transfection efficiencies, preparation of nuclear extracts and EMSA were essentially performed as described previously (22) (20). For testing BRCA1 promoter response to β -estradiol, MCF-7 cells were maintained in medium containing charcoal stripped serum purchased from Hyclone for at least three days (to deplete endogenous cellular estrogen). To elicit a proliferative response, MCF-7 cells were treated with 10^{-7} M β -estradiol.

Protein purification- The Py/Pu binding proteins were enriched from 293-cell nuclear extracts using affinity purification adapted from previously described procedures (23) (24).

RESULTS

Domains of the positive regulatory region (PRR)-

Schematic representation of PRR on the promoter and also the domains of the PRR are indicated in Fig. 1. Previously, the role of PRR in regulating BRCA1 promoter activity was described (20). Deletion of the 36 bp DNA segment consisting of the PRR resulted in a significant loss of the BRCA1 promoter activity. Additional investigations were initiated to characterize the BRCA1 PRR.

Sequence analysis of the PRR revealed that two potential domains exist within the PRR (Fig. 1). The 5' domain consists of over 96% homopyrimidine/homopurine (Py/Pu), wherein 26 out of 27 bases are Py/Pu. Interestingly, the domain has a symmetric structure, and the 26 Py nucleotides (when the sense strand is viewed) are evenly distributed on either side of the purine nucleotide, guanine. The second domain contains the consensus-binding site for the cyclic AMP response element binding (CREB) protein. In addition, our previous mutation studies indicated that sequential deletion of the Py/Pu and the CREB

domain resulted in a stepwise loss of BRCA1 promoter activity (19). These data strongly suggested that the Py/Pu and CREB sites acted synergistically or in concert to regulate BRCA1 transcription and indicated a bipartite nature of the PRR structure.

The prospect of a bipartite constitution of the PRR was tested by electrophoretic mobility shift assay (EMSA). The protein binding activities of the Py/Pu and CREB sites were assayed when both were present as a single unit (the PRR) as well as discrete sites (Fig. 2). The PRR site bound factors, which were detected as diffuse DNA-protein complexes (Fig. 2a; lane 2). Non-specific competition had no effect on the protein-DNA complexes (lane 3). Specific competition with an excess amount of double-stranded, unlabelled PRR abrogated the protein-DNA complex (lane 4). Competition with excess Py/Pu oligonucleotides resulted in decreased intensity of a higher mobility DNA-protein complex (arrow; lane 5). Similar competition studies with the CREB site resulted in a decrease in the lower mobility band (long arrow; lane 6). These experiments indicated that: (1) The proteins binding the PRR have specific affinity, (2) the higher mobility complex contained proteins with affinity for the Py/Pu site and that (3) the lower mobility complex contained proteins which bound the CREB site.

Experiments involving EMSA with discrete DNA probes derived from Py/Pu and CREB sequences were performed to further characterize the factors that bound these sites individually. With regard to the Py/Pu tract and the CREB site we observed a distinct, specific band for each probe (Fig. 2 c and d), although the CREB binding protein exhibited variable intensity of binding, perhaps reflecting regulation of its expression or processing. The CREB binding was also observed in Fig. 3b with the deletion mutants of the PRR with disrupted Py/Pu sites and intact CREB sites (-186 to -178).

The data indicated that specific factors bind the Py/Pu domain and the CREB site respectively

Transcriptional activity of the Py/Pu mutants-

Systematically deleted mutants of the PRR site on the BRCA1 promoter were transfected in MCF-7 cells and the promoter activity was measured. It was observed that there is a dramatic loss of activities with systematic deletion of bases.

Electrophoretic mobility shift assay (EMSA) with progressively deleted PRR probe revealed a gradual decline in the affinity of the probe for the binding proteins. The decrease in the binding affinity

was symmetric with the loss of promoter activity. These experiments reinforce the importance of the intact Py/Pu domain for the normal activity of the BRCA1 promoter.

Transfection of the point mutants revealed the bases that are potentially important for transcription. The 5' end as well some of the core bases in the PRR region were important for normal transcription of BRCA1 (Fig. 4a and b). BRCA1 promoters with altered Py/Pu sites demonstrated decreased transcriptional activities (Fig. 4a). Alteration of these bases resulted in a significant loss of luciferase activity.

Preliminary experiments with mutated CREB site also resulted in a loss of transcriptional activity. Therefore, both the Py/Pu domain as well as the CREB site were found to be essential for transcriptional activity of BRCA1 promoter.

PRR mediated effect of estrogen on BRCA1 transcription

MCF-7 cells, which were maintained in stripped serum for at least 3 days, were transfected with BRCA1 promoter constructs with intact and disrupted PRR (Fig. 5). On addition of physiological amounts of β -estradiol, a modest though reproducible increase of the BRCA1 promoter (with intact PRR) activity was observed. In contrast, there was no estrogen response by the BRCA1 promoter lacking intact PRR.

Enrichment of Py/Pu binding factors-

Affinity purification using the Py/Pu oligomers as a bait was used to purify the binding factors. The Py/Pu binding activity eluted in 2M NaCl. We are scaling up the procedure in order to obtain adequate quantities of the protein to enable identification by protein sequencing.

KEY RESEARCH ACCOMPLISHMENTS

- BRCA1 PRR is bipartite with a Py/Pu domain and CREB site.
- The estrogen effect on BRCA1 transcription is mediated by the PRR.
- Factors binding the Py/Pu domain have been enriched several hundred fold.

REPORTABLE OUTCOMES

A manuscript based on the results is being prepared for publication.

CONCLUSIONS

The results of the research is contributing to the understanding, with regards to the downregulation of the BRCA1 expression frequently observed in breast cancer cells. Elucidation of the factors (both *cis* and *trans*), involved in BRCA1 transcription, may contribute to the understanding of the pathology of breast cancer.

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APPENDICES

Figures and Figure Legends:

Fig. 1. Schematic representation and sequence of the of the Positive Regulatory Region (PRR) of the BRCA1 promoter. The Py/Pu and the CREB sites are indicated. The single purine base (G) in the Py/Pu site is indicated with an arrow and the 13 pyrimidine bases flanking the G are marked.

Fig. 2. PRR has two distinct functional domains. a) EMSA with probes designed from the PRR. The PRR probe used is indicated in Fig. 1. Competitions were performed with 100 fold excess of double-stranded, annealed and unlabeled PRR, Py/Pu (5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT-3') and CREB (5'-CCT TTT ACG TCA TCC GGG GGC AGC T-3') oligomers. EMSA with (c) Py/Pu and (d) CREB probes were also performed. The non-specific competitor used was a synthetic oligomer, 5'-GTC ACT ATG GCT TTC AAT TGG CCC GGC ATA G-3' annealed to its complementary sequence.

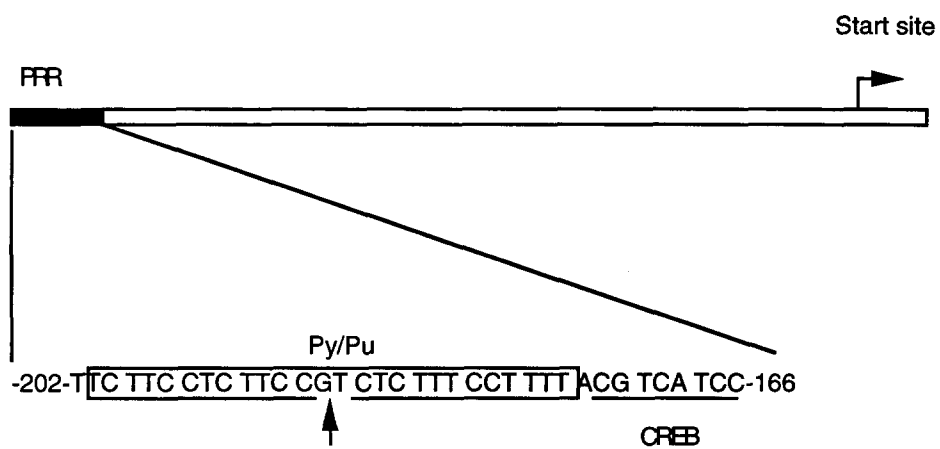
Fig. 3. Analysis of the deletion mutants of the PRR. (a) Transcriptional activities of the systematic deletion mutants of the Py/Pu domain. The 5' deletion mutants as well as the normalized luciferase activities are indicated. (b) ~~DNA binding analysis of the systematically deleted PRR.~~

Fig. 4. Point mutants of the PRR. (a) Point mutants of the PRR were generated by PCR and subcloned in the pGL3 basic vector. The transcriptional activities (as measured by luciferase activities) of each mutant is indicated.

Fig. 5. Estrogen response of BRCA1 transcription is mediated by the PRR region. (a) The positive response to estrogen by BRCA1 promoter is mediated by the PRR. MCF-7 cells were transfected with BRCA1 promoters with intact PRR (-202) and disrupted PRR (-180). The transfected cells (which were maintained in media with stripped serum) were treated with 10^{-7} M β -estradiol where indicated. Normalized luciferase activities are indicated.

Fig. 6. Enrichment of Py/Pu binding proteins. Approximately 29, 750 mL flasks of 293 cells were utilized to make nuclear extracts as previously described (20). A column containing agarose beads linked to Py/Pu DNA (prepared as described in [1996 #1234]) was used to enrich a partially purified extract (utilizing size fractionation; data not shown) (a) The partially purified sample was passed over the column (by size fractionation; data not shown) and washed with increasing salt concentration.

Fig. 1



CREB competitor	-	-	-	-	-	+
Py/Pu competitor	-	-	-	-	+	-
PRR competitor	-	-	-	+	-	-
NS competitor	-	-	+	-	-	-
MCF-7 N.E.	-	+	+	+	+	+
PRR probe	+	+	+	+	+	+

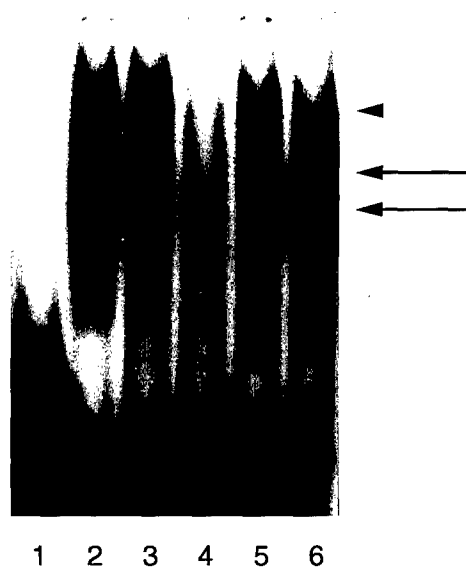
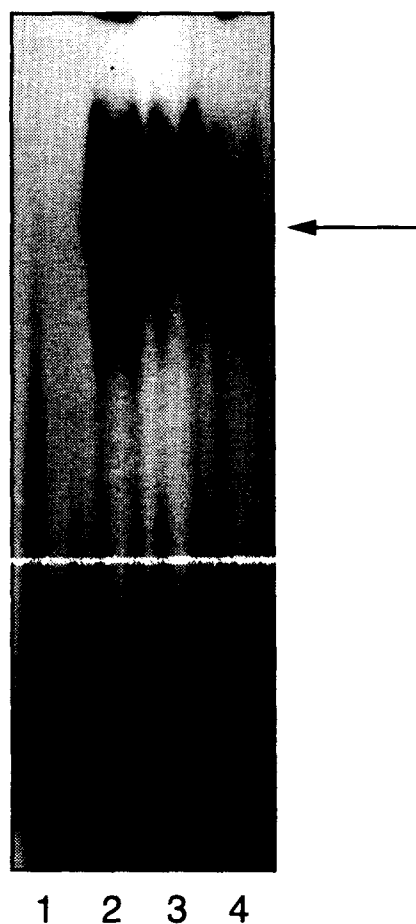
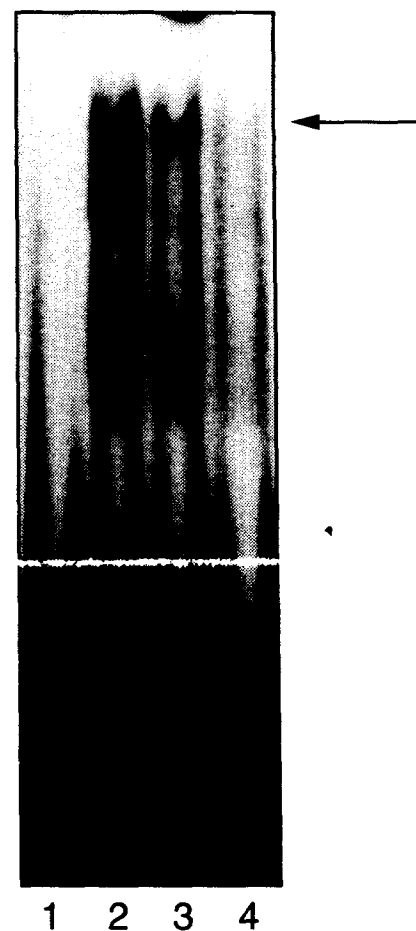


Fig. 2B

S	-	-		+
NS	-	-	+	-
MCF-7 N.E.	-	+	+	+
Py/Pu probe	+	+	+	+



S	-	-		+
NS	-	-	+	-
MCF-7 N.E.	-	+	+	+
CREB probe	+	+	+	+



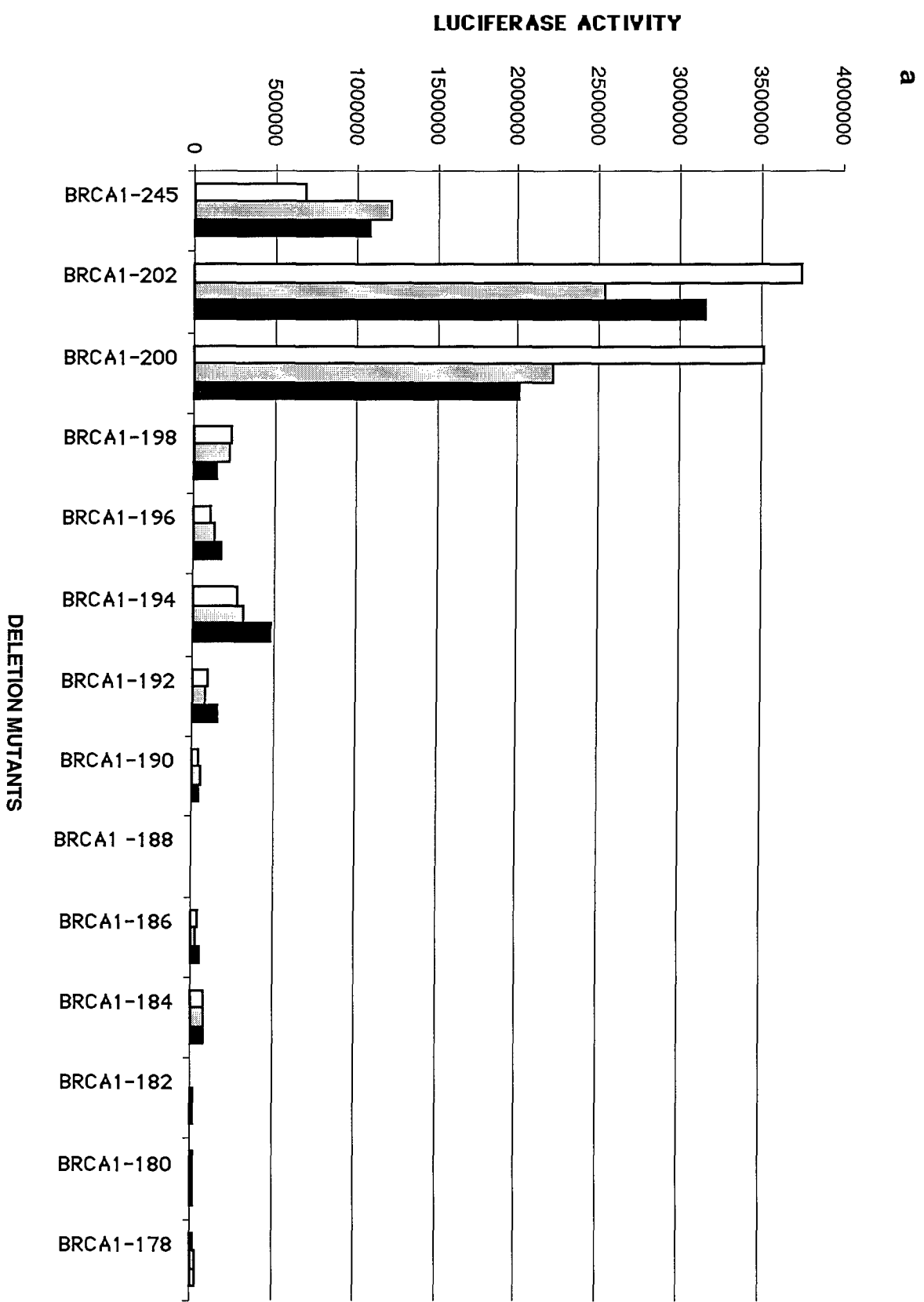
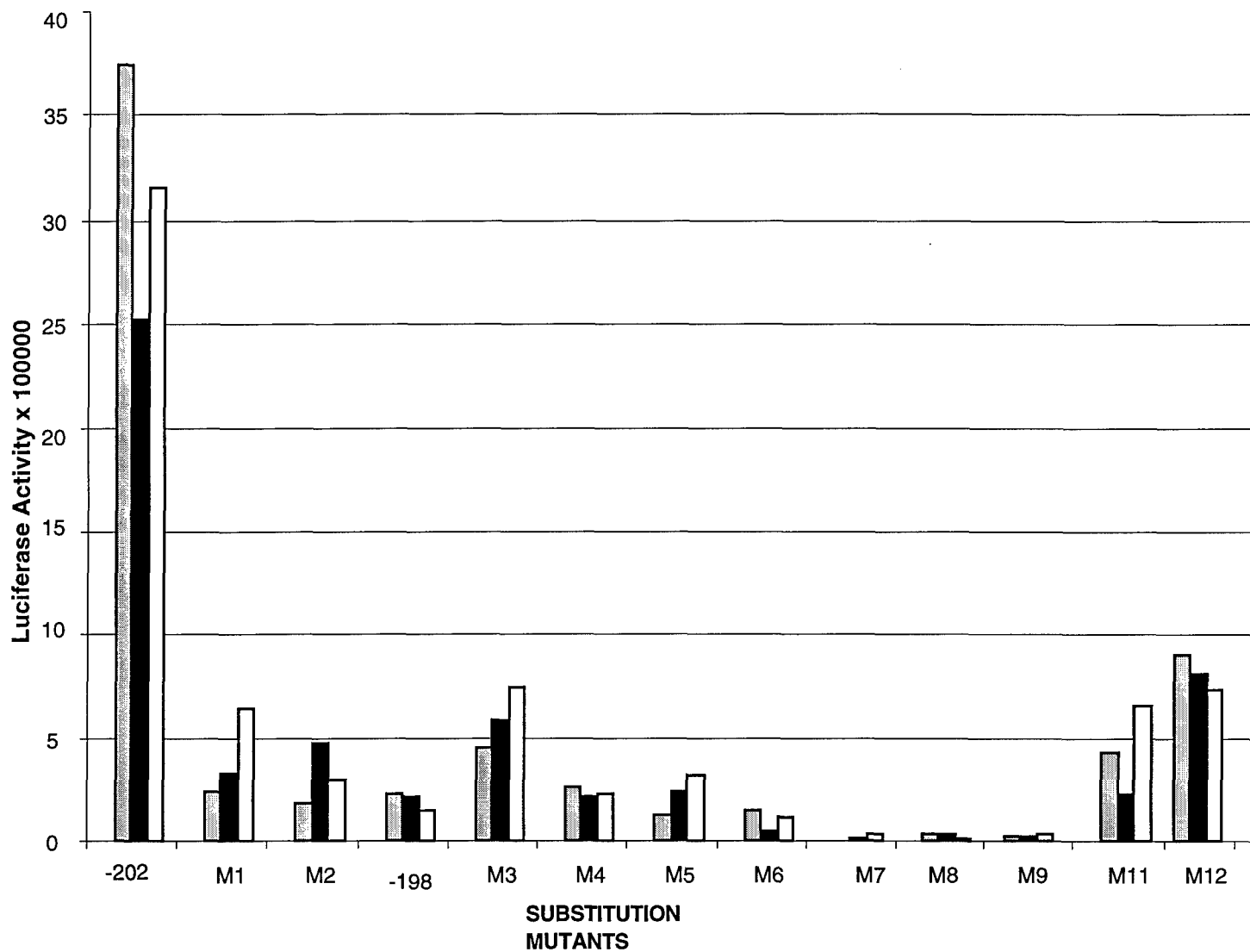


Fig. 4



1 -202 wt	TTC TTC CTC TTC CGT CTC TTT CCT T
2 -202 M1	AGC TTC CTC TTC CGT CTC TTT CCT T
3 -202 M2	TTA GTC CTC TTC CGT CTC TTT CCT T
4 -198 wt	TC CTC TTC CGT CTC TTT CCT T
5 -198 M3	AG CTC TTC CGT CTC TTT CCT T
6 -198 M4	TC AGC TTC CGT CTC TTT CCT T
7 -198 M5	TC CTA GTC CGT CTC TTT CCT T
8 -198 M6	TC CTC TAG GTC CTC TTT CCT T
9 -198 M7	TC CTC TTC AGT CTC TTT CCT T
10 -198 M8	TC CTC TTC CAT GTC CTC TTT CCT T
11 -198 M9	TC CTC TTC CGA GTC TTT CCT T
12 -198 M11	TC CTC TTC CGT CTC AGT CCT T
13 -198 M12	TC CTC TTC CGT CTC TTA GCT T

Fig. 5

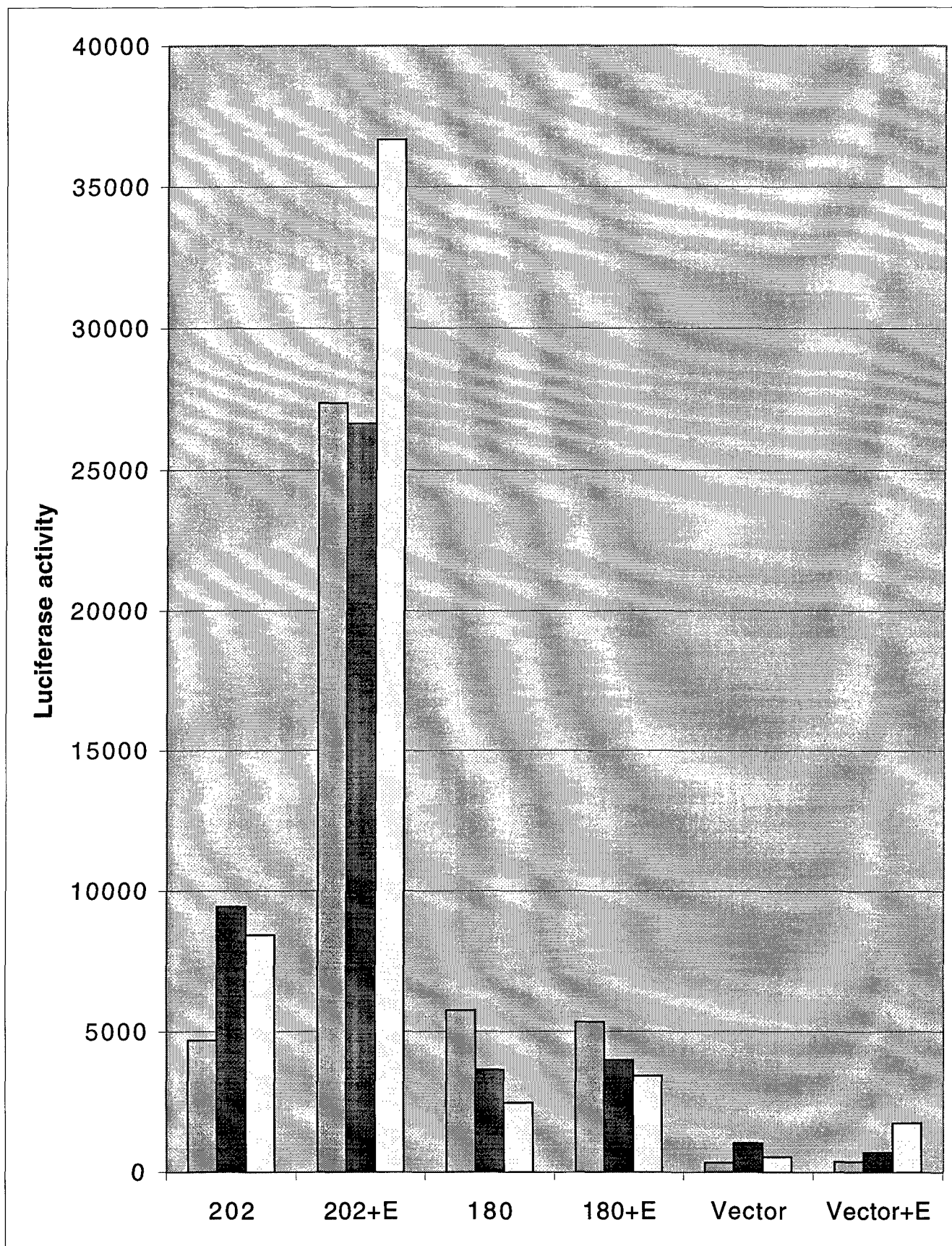
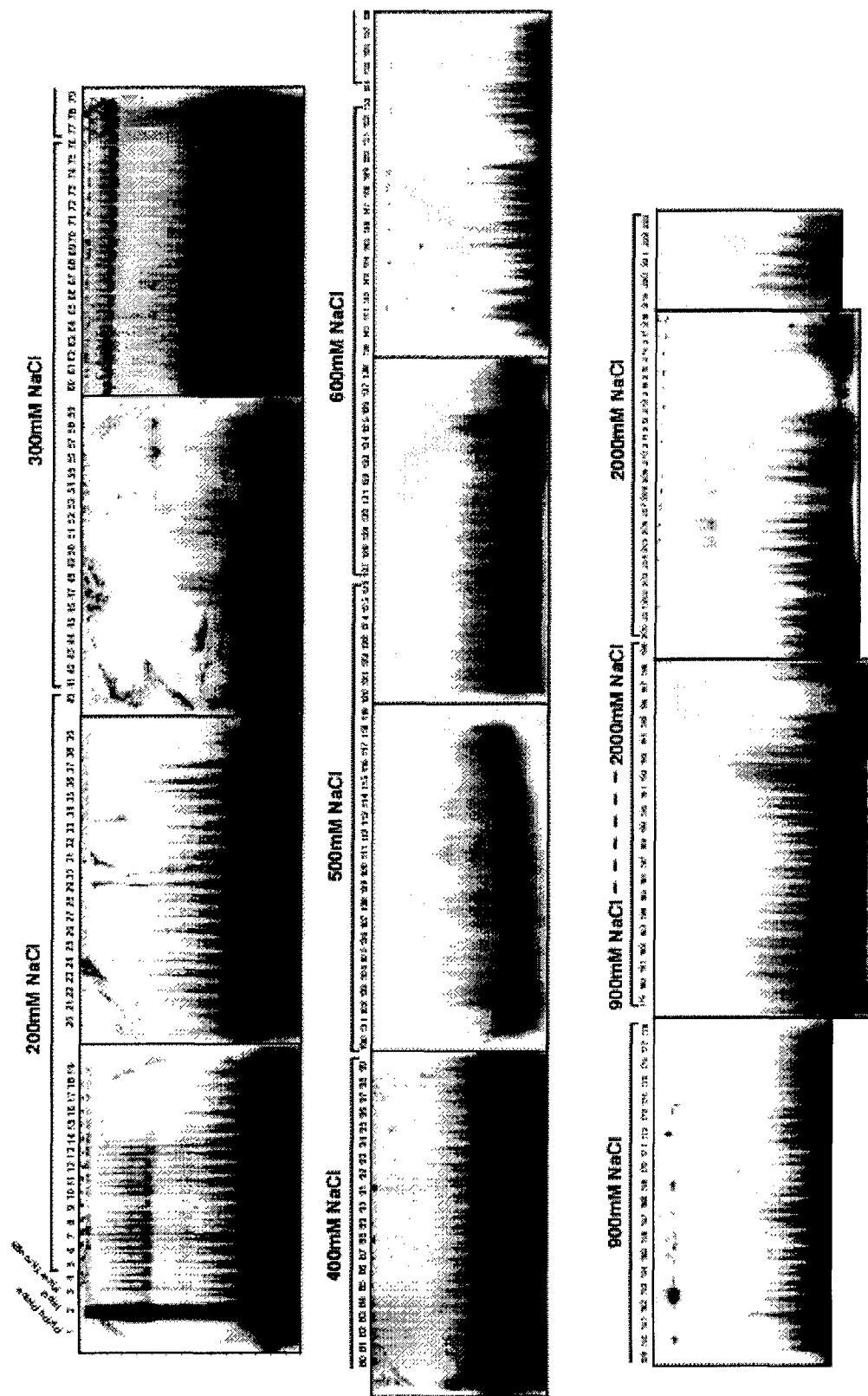


Fig. 6





DEPARTMENT OF THE ARMY

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504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLIS M. RINEHART
Deputy Chief of Staff for
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