AD_____

GRANT NUMBER DAMD17-94-J-4053

TITLE: A Role for the NF-kb/Rel Transcription Factors in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Albert S. Baldwin, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
Chapel Hill
Chapel Hill, NC 27599-7030

REPORT DATE: July 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Final (1 Jul 94 - 30 Jun 98)

4. TITLE AND SUBTITLE
A Role for the NF-kb/Rel Transcription Factors in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4053

6. AUTHOR(S)
Albert S. Baldwin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

19990323 113

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Human breast cancer is characterized by the inappropriate expression of growth factors, kinases and possibly certain transcription factors. Our project has focused on the regulation of the NF-kB family of transcription factors in human breast cancer. Additionally, the project has analyzed a role for the VNTR elements in the hereditary susceptibility of women to breast cancer. We have found that NF-kB is activated in virtually all breast cancers. Interestingly, the form of NF-kB that is activated is not the classic form of NF-kB (the p50/p65 heterodimer) but rather involves the activation of the p52 form of NF-kB along with c-Rel and the IkB homolog Bcl-3. Additionally, our work revealed that NF-kB suppresses transformation associated apoptosis and apoptosis induced by chemotherapy and radiation. We have identified a DNA binding activity that binds to the VNTR element. The possible association of the tumor suppressor protein p53 with this element is currently being explored. Furthermore, our data indicate that African-American and white controls differed in the normal spectrum of VNTR alleles. Rare alleles were significantly associated with breast cancer in African-American women, but not in white women.

14. SUBJECT TERMS	15. NUMBER OF PAGES
H-Ras VNTR, Genetic Susceptibility, NF-kb/Rel Proteins, Transcription Factors, Gene Regulation, Molecular Markers, Humans, Anatomical Samples, Breast Cancer	91
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Albert J Baldwin 9/16/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION:

Human breast cancer is characterized by altered expression of growth factors, growth factor receptors and kinases (Lippman and Dickson, 1989). Downstream modulators of growth factors and kinases are transcription factors which likely modulate altered responses in breast cancer. Genetic analysis also indicates that other factors are involved in development or progression of human cancer, including repetitive DNA elements called VNTR elements (Krontiris et al, 1993), the recently cloned BRCA1 gene (Futreal et al, 1994), and the tumor suppressor gene p53 (Rotter et al, 1993). Additional emphasis has been placed on the role of estrogen in the development of breast cancer.

NF-κB/Rel proteins are widely distributed and are typically found in the cytoplasm where they are associated with an inhibitor protein called IκB (Beg and Baldwin, 1993; Baldwin, 1996). Upon exposure to inflammatory cytokines or growth factors, NF-κB factors dissociate from the IκB inhibitory proteins and translocate into the nucleus (Finco and Baldwin, 1995). In the nucleus, these dimeric factors regulate transcription of genes that contain the κB binding site. Typically these genes encode proteins involved in immune and inflammation responses but more recent work has shown that genes encoding growth regulatory proteins (such as c-*myc*) are regulated by NF-κB (see Baldwin, 1996). There are presently five members of the NF-κB/Rel family of proteins: p50/NF-κB1, p52/NF-κB2, c-Rel, RelB, and p65/RelA. There are four members of the IκB family: IκBα, IκBβ, IκBε, and Bcl-3. The first 3 forms of IκB function as inhibitors while Bcl-3 appears to function as a transcriptional co-activator with the p50 and p52 subunits of NF-κB (see Results section).

NF-κB and IκB proteins are associated with oncogenesis. For example, members of the NF-κB family of proteins are related to the product of the c-Rel proto-oncogene which is found overexpressed in certain tumor cell lines. Additionally, the p100/NF-κB2 member of the NF-κB family is found translocated in certain lymphomas as is Bcl-3, a member of the IκB family (see Beg and Baldwin, 1993; Baldwin, 1996). Based on our preliminary data, we have proposed that dysregulation of normal NF-κB regulation (i.e., chronic nuclear localization of some forms of NF-κB) may play an important role in the development or progression of human breast cancer. However, new data indicates a second mechanism whereby NF-κB may be activated and separate new data indicates a mechanism whereby NF-κB appears to function in oncogenesis (see Progress/Results section). These new results take on broadly significant implications regarding oncogenesis in general and particularly in regards to breast cancer since it was published last year that oncogenic HER2/Neu (an oncogene activated in a significant number of breast cancers) can activate transcription through NF-κB binding sites (Galang et al., 1996). In fact, our recent data indicate that NF-κB is required for oncogenic transformation and tumorigenesis (see progress/results section).

We have previously found that a breast cancer cell line exhibited constitutive activation of NF-κB. Furthermore, we had found that estrogen treatment of a fibroblast caused enhanced expression of a reporter gene that is controlled by multiple NF-κB sites. Furthermore, some genes shown to be overexpressed in human breast cancer (for example, vimentin and ICAM-1) are known to be regulated by NF-κB. Thus one broad aim of the proposal (Dr. Baldwin's part of the project) was to study the expression of NF-κB/Rel proteins in human breast cancer and to study the functional outcome of this potential overexpression. Our new findings do not alter the aims that were originally planned but they do alter how we approach them (see below). Additionally, these results raise questions regarding whether oncogenes associated with breast cancer (HER2/Neu or TC21) may functionally target NF-κB as part of the transformation process.

A second focus of this proposal is the functional study of the Ha-ras variable number tandem repeat (VNTR) in breast cancer. VNTRs arise from the head to tail concatenation of short sequence motifs. The VNTR at the Ha-ras locus is about 1 kilobase downstream from the Ha-ras 1 proto-oncogene and consists of 30-120 copies of a 28 bp sequence. Krontiris et al (1987) first showed that rare alleles of Ha-ras appear in the genome of cancer patients at a higher frequency

that in non-affected individuals. More recent data indicate that the breast cancer risk due to a rare allele may be greater in African-American women (Garrett et al, 1993).

It has been published that the Ha-ras VNTR appears to bind NF-κB transcription factors, although supershift studies were never performed to verify this protein binding (Trepicchio and Krontiris, 1992). In addition, a number of studies have reported transcriptional enhancement activity of this VNTR (Green and Krontiris, 1993). The broad aims of this part of the proposal (Dr. Conway) are to explore a role for VNTR elements in human breast cancer, evaluate the association of rare alleles with breast cancer and further characterize the factors that bind to the Ha-ras VNTR element.

As stated above, rare alleles of the Ha-ras VNTR occur more frequently in individuals with cancer, including breast cancer, than in those without cancer (Garrett et al., 1993; Krontiris, 1994). Although the mechanism of this is association is unknown, one explanation may be that Ha-ras alleles possess a biological function and the rare alleles may function differently than the common alleles. If the Ha-ras VNTR possesses transcriptional regulatory activity, this function might be affected by variations in allelic sequence. We have found that the various Ha-ras alleles vary not only in repeat copy number but also in the interspersion pattern of repeat sequences along the VNTR. Sequence analysis of individual 28 bp repeats shows that a given repeat may possess either a G or a C at positions 7 or 15, giving rise to four possible repeat types. We have developed a minisatellite variant repeat (MVR)-PCR approach to detect these sequence variants. Using this method, we have shown that VNTR allele length is very tightly linked to MVR internal sequence (Conway et al., 1996). That is, nearly all alleles having the same length also have the same MVR allelotype. In contrast to the common alleles that are detected repeatedly in a population, rare alleles possess unique and disordered sequences (Conway et al., 1996; Conway et al, submitted). Most rare VNTR alleles begin at the 5' end as one common allele then abruptly switch to either a second recognizable motif or become completely unrecognizable. This suggests that rare MVR alleles arise via recombination involving segments of one or more of the common alleles.

The G/C polymorphisms within the 28 bp repeat subunit could potentially affect transcription factor binding, perhaps by influencing methylation patterns or DNA secondary structure. If we are able to more clearly define the differences between rare and common alleles based upon a combination of transcription factor binding, transcriptional enhancement activity, and structural characteristics such as VNTR length and internal sequence variations, we can identify true rare alleles which predispose to breast cancer. To this end, we proposed to investigate the potential regulatory role of the Ha-ras VNTR by characterizing its interaction with members of the NF-κB/Rel family and other transcription factors. This was to be accomplished by evaluating protein binding to 28 bp VNTR subunits carrying specific G/C polymorphisms, and to longer tandem arrays of subunits generated by MVR from either common or rare alleles. We were also interested in characterizing the binding proteins present in a series of nuclear extracts from cell lines and from breast tumor tissues as well as normal mammary epithelium. Finally, we wanted to determine the biological outcome of VNTR/protein interactions by characterizing transcriptional regulatory activity of common versus rare VNTR sequences. Listed below are the original aims of the grant application:

Aim 1 is to investigate the potential biological function of the Ha-ras VNTR through characterization the nuclear factors that bind to this element with a definite focus on the potential interaction of NF-κB/Rel proteins. Further approaches include studies aimed at addressing potential transcriptional activation properties of the VNTR.

Aim 2 is designed to determine if NF-κB/Rel binding to VNTR elements may be used as a more refined method of identifying patients at risk for breast cancer.

Aim 3 is to analyze relative nuclear and cytoplasmic levels of NF-κB/Rel proteins in normal breast epithelium and in human breast cancer. We will correlate NF-κB expression with activation of certain kinases thought to regulate NF-κB expression and with the status of transcriptional activators shown to regulate NF-κB gene expression.

Aim 4 is to correlate expression of NF-κB with expression of known or suspected prognostic markers for human breast cancer (ICAM-1, urokinase and vimentin). We will determine if the ligand for HER2/Neu, NDF, can induce the expression of NF-κB.

Aim 5 is to determine whether estrogen can regulate gene expression through a κB site and whether this is due to the activation of NF-κB/Rel binding activity.

BODY (Baldwin Laboratory):

Progress/Results (some of these results were described in the last report but papers have now been published/submitted and are now described in more detail):

NF-κB functional activity is controlled by Ras oncogenic alleles. We have initiated our studies using the oncogene Ras and its downstream effector Raf. We show (see Finco et al., attached) that oncogenic Ras or oncogenic Raf potently activates gene expression through an NF-κB binding site. However, oncogenic Ras or Raf do not increase nuclear translocation of NF-κB. This presented an enigma to us: how is it that NF-κB functional activity can be increased without an increase in nuclear NF-κB? So we tested whether the innate transcriptional activity of NF-κB p65 is increased under these conditions. Our experiments indicate that the transcriptional activation domain of the p65 subunit of NF-κB is functionally targeted by oncogenic Ras or oncogenic Raf. Thus, these data indicate that two oncogenes (Ras or Raf) can potently stimulate transcription of NF-κB dependent transcription without inducing nuclear translocation of NF-κB. This is accomplished apparently by the targeting of the transcriptional function of the p65 subunit which exists at low, constitutive levels in most cells.

NF-κB is required for oncogenic Ras to neoplastically transform cells. With the above data in mind, we have asked whether NF-κB is required for oncogenic Ras to transform cells. We now show that the ability of Ras to transform cells is blocked by expression of the NF-κB inhibitor IκBα (see Finco et al., in the appendix). Additionally, we show that cells that have the NF-κB p65 genes deleted are incapable of activation of κB-dependent transcription in response to oncogenic Ras or oncogenic Raf. Thus, these data indicate that the NF-κB subunit p65 is required for oncogenic Ras to transform cells. We are obviously very interested in determining if transformation by the breast cancer oncogene HER2/Neu requires NF-κB. This question will be addressed in future experimentation.

NF-κB functional activity blocks apoptosis. Protection from apoptosis is a critical component in neoplastic transformation and in protection from radiological and chemotherapies (Fisher, 1994). Our data now indicate that NF-κB activation protects cells from killing by several cancer therapies. Data shown in the attached manuscript (Wang et al, 1996) indicates that NF-κB protects cells against killing by tumor necrosis factor. The experimental approach was to express a super-repressor form of IκBα (mutated in serines 32 and 36, which results in a protein that can bind to NF-κB but which cannot be degraded) in the HT1080 fibrosarcoma cell line. This cell line was chosen since it is highly resistant to killing by TNF. Expression of IκBα blocks NF-κB nuclear translocation in these cells and correspondingly sensitizes to killing by TNF. Further evidence (not shown) that NF-κB is protective against killing by TNF are the following observations: (1) re-expression of NF-κB subunits in the cells expressing IκBα restores protection against killing, (2) fibroblasts that are null for the p65 NF-κB subunit (from the p65 knockout) show enhanced killing by TNF, and (3) cells that are highly sensitive to killing by TNF do not activate NF-κB in response to this cytokine. Thus, these data strongly indicate that NF-κB protects cells against killing by TNF.

The requirement of NF-κB for Ras-mediated transformation is based on the anti-apoptotic function of NF-κB. The ability of IκBα expression to block Ras-mediated cellular

transformation (described above) appears to be based on the ability of NF-κB to block apoptosis. As described in Mayo et al (see attached manuscript), inhibition of NF-κB in the setting of expression of oncogenic Ras leads to apoptosis. This result has important implications for our data described below, suggesting that NF-κB activation in breast cancer may serve as an anti-apoptotic function to prevent oncogene-induced apoptosis.

NF-κB is activated by cancer chemotherapies and radiation and significantly blunts the apoptotic response to these therapies. Since several cancer chemotherapies kill transformed cells by the induction of apoptosis (Fisher, 1994), we have determined if NF-κB protects against killing by these treatments. We have initially focused on 2 standard cancer therapies: ionizing radiation and daunorubicin. Our data (see Wang et al., 1996, manuscript attached) indicate that the inhibition of NF-κB potently enhances cell killing in response to radiation and daunorubicin treatment. Interestingly, radiation and daunorubicin activate NF-κB but adriamycin does not. Thus, our data indicate that cancer therapies that kill and also activate NF-κB are (at least partially) less effective at inducing apoptosis unless NF-κB activity is blocked. Recent data indicate that camptothecin/CPT-11 activates NF-κB and the inhibition of NF-κB significantly improves the tumor cell killing response. Thus, we propose that inhibitors of NF-κB will potently enhance cell killing by ionizing radiation and daunorubicin (and potentially other therapies).

Analysis of NF-κB expression in human breast cancer: evidence for increased nuclear localization of p50 and p52 NF-κB subunits and Bcl-3. We have largely accomplished one major aspect of Aim 3; that is, analyzing the expression of NF-κB/Rel proteins by immunohistochemical studies of human breast cancer sections. Utilizing the antibody against the human NF-κB p65 subunit, it was found that approximately 30-35% of human breast tumors express extraordinarily high levels of NF-κB, as compared to normal breast epithelium (reported in the last report). Interestingly, the majority of these exhibit cytoplasmic levels of p65, although NF-κB p65/RelA is nuclear in several examples.

Our recent data on nuclear extracts of breast tumors shows that there is a significant increase in NF-κB binding activity in the tumors versus the adjacent tissue controls (see Cogswell et al, in the Appendix). We noted that the mobility of this form(s) of NF-κB was faster than authentic NF-κB p50/p65. Antibody supershift data indicate that p50 and p52 were included in the gel mobility shift complex (see Figure 2 in the appendix). Additionally, immunoblotting of nuclear extracts versus adjacent tissue controls showed that NF-κB p50, p52 and c-Rel were all expressed in the nucleus of breast cancer cells relative to the adjacent tissue controls (see Cogswell et al., in the appendix).

BODY (Conway Laboratory):

H-ras VNTR allele length typing by PCR H-ras VNTR alleles were characterized in 157 incident breast cancer cases and 198 unaffected controls, using a combination of PCR allele length and 5' MVR sequence methods (see Appendix, Conway et al, submitted). PCR demonstrated that a cluster of alleles, differing in length by one or a few repeats, occurred around each common allele mode. The modal common allele lengths identified by PCR was 30 repeats for a1, 46 repeats for a2, and 69 repeats for a3. Several alleles ranging from 85 to 88 repeats were identified for a4. The allele length distributions were found to differ between the African-American and white subjects; whites possessed the 4 previously described alleles a1, a2, a3 and a4, while blacks possessed primarily a1 and a4-related alleles.

Internal sequence variation in the H-ras VNTR identified by minisatellite variant repeat (MVR)-PCR demonstrates that sequence is associated with allele length The H-ras VNTR 5' sequences were characterized by MVR-PCR (see Appendix, Conway et al, 1996). H-ras allele length was found to be tightly linked with MVR sequence for each of the H-ras common alleles

such that each allele possesses its own characteristic, distinguishing 5' MVR sequence. The alleles with A1a and A1b MVR patterns are typically 30 repeats in length, while the A2 MVR pattern is 46 repeats in length. Three patterns, A3a, A3b and A3c, were associated with the a3 common allele of 69 repeats, while one A4 pattern was found in the a4 allele group of 85-88 repeats. Four additional distinct MVR patterns were identified for alleles previously classified as intermediate frequency alleles, a1-2 (28 repeats), a1+2 (32 repeats), a1+4 (34 repeats), and a1+6 (36 repeats). MVR also demonstrated some minor variation in sequence even within the common alleles. Many rare alleles possessed disorganized 5' sequences, suggesting their derivation by some recombinatory mechanism. Furthermore, the 5' MVR sequence indicated the allelic origin of each rare allele. MVR complemented the length analysis and led to an increased detection of rare alleles by enabling us to identify structurally abnormal alleles with common lengths.

Rare H-ras alleles and breast cancer risk The risk of breast cancer associated with rare H-ras alleles was affected by the length and sequence criteria used to define rarity (see Appendix, Conway et al, submitted). The breast cancer risk associated with a rare H-ras allele was substantially elevated in black breast cancer patients, and this result was significant when rarity was defined by length alone, by MVR alone, or by a combination of these criteria (OR=5.6, 95% CI=1.2-25.8). In contrast to earlier studies using Southern blotting technology, an increase in breast cancer risk was not found in whites using any of the PCR-based rare allele classification approaches, however, a specific rare allele of 59 repeats in length occurred more frequently in white breast cancer patients (OR=5.3, 95% CI=0.5-67.7), but this finding was not statistically significant due to the low prevalence of these alleles.

Protein interactions with the Ha-ras VNTR evaluated by gel mobility shifts We have characterized the interaction of the Ha-ras VNTR with transcription factors, including those of the NF-κB/rel family, using gel mobility shift assays. We have synthesized double-stranded 28 bp oligonucleotides corresponding to each of the 4 Ha-ras VNTR subunit repeat types carrying specific G/C polymorphisms. Oligonucleotides BstN-1 (with polymorphisms G/C), BstN-2 (C/C), BstN-3 (C/G) or BstN-4 (G/G) were ^{32}P-end-labeled and were incubated with nuclear protein extracts derived from a series of cell lines (MCF-7 breast tumor, T47D breast tumor, SKBR3 breast tumor, BT20 breast tumor, Jurkat T cell leukemia, K562 leukemia, and HeLa cervical carcinoma), then were run in non-denaturing polyacrylamide gels to compare the protein binding patterns among the various cell lines and to determine which repeat type was bound most tightly by nuclear proteins. Cells remained untreated or were treated (induced) with phorbol myristate acetate (PMA), phytohemagglutinin (PHA), TNF-α or a combination of PMA and PHA.

The VNTR sequences exhibited protein binding profiles that appeared to correspond to NF-κB proteins. Two bands of similar size were visible in all cell lines when a single copy of the VNTR repeat probes (BstN1-4) were used. Binding to the VNTR sequence was the same for both the PMA-induced and the uninduced extracts. This failure by PMA induction to produce any additional protein binding suggests that the inducible form of NF-κB, p65, does not bind the VNTR. Protein binding to the VNTR sequence was completely inhibited at both bands by the unlabeled VNTR probes, and was also inhibited by the unlabeled NF-κB (UV-21 or Ab') concensus sequences, with competition strongest at the upper band. In addition, protein binding to the VNTR probe was inhibited by an unlabelled p53 concensus DNA-binding sequence (mainly in the lower band), only slightly by an Apo-B VNTR sequence, but not by an SRE concensus. As previously shown, the AP-1 concensus sequence also competes for protein binding preferentially at the lower binding band of the Ha-ras VNTR. The results of these experiments suggest that several proteins which recognize NF-κB, AP-1 and p53 concensus DNA binding sequences also bind the Ha-ras VNTR. Because two main bands of VNTR binding occur, and each can be competed with a different sequence, there is likely more that one protein and possibly a multi-protein complex binding to the VNTR.

Control studies evaluating NF-kB protein binding to its concensus sequence In order to be sure that nuclear NF-κB proteins were being induced in cells by the PMA treatments, we also performed binding experiments with a labeled NF-kB (UV-21) concensus oligonucleotide. In contrast to the Ha-ras VNTR which did not show increased protein binding in response to PMA stimulation, "classical" inducible NF-κB protein (p65) binding to the NF-κB (UV-21) sequence was observed with the PMA- and TNF-α treated extracts, and the p65-DNA complex was supershifted using p65 antisera. Supershifting of the p50/UV-21 complex was also observed with the p50 antibody. This NF-κB binding was competed by the cold NF-κB and Ha-ras VNTR sequences. Our experiments suggest a lack of classical NF-κB/p65 binding to the VNTR; interestingly, Dr. Baldwin's lab has shown that increased NF-κB/p65 detected in most breast tumors by immunohistochemistry remains in the cytoplasm.

Comparison of protein binding to the H-ras VNTR versus the p53 consensus sequences We have also evaluated the potential interaction of the p53 tumor suppressor protein with the Ha-ras VNTR. The Ha-ras VNTR repeat types 3 and 4 (corresponding to the 28 bp BstN-3 and 4) possess p53 half-sites and the potential binding site lies at one of the G/C polymorphisms. P53 binds DNA in a sequence-specific manner and regulates transcription of a variety of genes (Funk et al, 1992; Friend, 1994), it is mutated or overexpressed in many breast tumors (Seth et al, 1990; Bartek et al, 1990), and, interestingly, p53 and NF-κB appear to have opposite effects on apoptosis. Dr. Baldwin's lab has shown that NF-κB inhibits apoptosis.

For the p53/VNTR binding studies, we used nuclear extracts from the breast tumor lines MCF-7 (wild-type but cytoplasmic p53) and T47D (mutant p53), SKBR3, and Jurkat, and single repeat oligonucleotide probes (BstN-1, BstN-3 or BstN-4) or the doublet of the VNTR repeat type 3. We have also used the p53 consensus DNA binding sequence as a positive control in order to determine the pattern of protein binding expected with p53 for comparison with that obtained with the VNTR. The p53 and Ha-ras VNTR probes exhibited the exact same two bands of protein binding (one major and one minor), regardless of the protein extract used. These bands appeared to be the same as those seen in previous DNA binding experiments using the VNTR, AP-1 and NF-κB (UV-21) probes. There was no effect of induction by PMA on the quantity of protein bound to the p53 or the VNTR sequences.

Supershifts suggest that NF-κB/p52 (and possibly p50) binds the Ha-ras VNTR In an effort to identify the proteins which bind the Ha-ras VNTR, we performed a series of gel supershifts using antisera specific for a number of transcriptional regulatory factors. These studies are summarized in Table 1. Supershifting using antibodies to the various NF-κB family members was performed using single VNTR repeats, a doublet of the VNTR type 3 repeat, and a 20-repeat VNTR fragment generated by MVR. Increasing the length of the target VNTR oligonucleotide from 1 to 2 repeats permitted the detection of supershifting or enhanced protein binding with antibodies to both the p52 and p50 forms of NF-κB. Some supershifting was noted with the 20-repeat MVR fragment but this experiment did not yield clean results with discrete bands due to the large size of the DNA sequence. The binding of p52 (and p50) to the Ha-ras VNTR are of interest in light of the findings of Dr. Baldwin's lab showing increased nuclear localization and binding activity of p50 and p52 NF-κB in breast tumor tissue extracts.

Enhanced binding/supershifting suggests that p53 binds the Ha-ras VNTR Some p53 antisera have been found to enhance or stabilize specific binding of p53 to DNA in gel mobility shift assays (Hupp et al, 1992). The PAb421 antibody enhances p53 sequence-specific DNA binding, and subsequent addition of the second D01 antibody may in some instances induce a supershift of this complex in gel mobility shift assays. However, many investigators, including Dr. Baldwin's lab, have reported difficulty in supershifting p53/DNA complexes. We observed enhanced binding of proteins to the Ha-ras VNTR sequence in the presence of the individual p53 antibodies PAb421 and D01, and possibly a supershift with the combination of antibodies as demonstrated by the loss of the band at the lower arrow in Figure 2, lane 5. The ability to

produce a supershift did not appear to be related to VNTR length, since supershifting was also seen with the single repeat type 3.

Table 1: Summary of Gel Supershift Studies to Identify Proteins Binding to the Ha-ras VNTR

Antibody	Protein	Supershifting Observed with		
		1 repeat	2 repeats**	20 repeats***
D01(Ab1)+PAb421(Ab6)	p53 (wildtype in MCF-7 cells*)	E(+)	E(+)	nd
SC109 (Baldwin lab)	NF-κB/p65 (induced form)	-	nd	nd
SC114 (Baldwin lab)	NF-κB/p50 (constitutive form)	-	E	+
Ab84	NF-κB/p52 (constitutive form)	-	+	+
SC253X, K-25	c-fos (AP-1)	-	nd	nd

ip; in progress, nd; not done, E; enhanced binding. *p53 in MCF-7 is primarily cytoplasmic, and only a small amount of supershifting is observed presumably from contaminating cytoplasmic protein in nuclear extracts. **2 tandem repeats of the type 3 repeat. ***MVR-generated fragment corresonding to the first 20 5' repeats of an A1a common allele sequence.

Transcriptional enhancement by the common VNTR alleles depends on allele length. In order to evaluate potential differential transcriptional regulatory functions of the Ha-ras VNTR alleles, we TA-cloned Ha-ras VNTR alleles (a1, a2, a4 common alleles of lengths 30, 46 and 87 repeats, respectively, and rare alleles #139 with 29 repeats, #13 with 43 repeats, and #348 with 81 repeats, into luciferase reporters downstream of both the SV-40 promoter and luciferase gene, which is the normal position of the Ha-ras VNTR with respect to the c-Ha-ras gene. The a1 and a2 common alleles are comprised of types 1, 2 and 3 repeats, while the a3 and a4 alleles also possess the type 4 repeat. Large-scale quantities of full length a3, a4 or large rare allele/luciferase constructs have been exceedingly difficult to obtain for transfections due possibly to the instability of these sequences in bacteria, thus our progress in this area has been severely hampered.

Transcriptional enhancement by the Ha-ras VNTR was evaluated in a number of different cell lines including the MCF-7 (normal but cytoplasmic p53), T47D (mutant p53), BT-20 (breast tumor), MCF-10 (wildtype p53, immortalized breast), and K562 (mutant p53). Transient transfections were performed as follows. Cells were plated and allowed to grow to 40-60% confluence, then were transfected with the construct of interest using Lipofectin reagent. The molar quantities of the plasmids transfected were adjusted to control for the varying lengths of the VNTR region. Cultures were allowed to grow overnight and the following morning, the medium was changed. After 48 hrs, cells were either untreated or were PMA-treated for 3 hrs. Media was changed again and the cells were harvested by lysis in 0.2 M Tris, pH 7.5 and three cycles of freezing and thawing. Cell extracts containing 10-100 ug of protein were placed in a luminometer, the substrates ATP and luciferin were simultaneously injected and light emission was measured. Luciferase expression is given in fold expression or relative light units (RLU).

Transfection of either untreated T47D breast tumor cells or K562 cells with the Ha-ras common alleles positioned downstream from the luciferase gene produced varying levels of transcriptional enhancement above the background level of vector alone. In PMA-treated T47D, the a1 (30 repeats) or a2 (46 repeats) common VNTR alleles led to a 7.5 and 6.5-fold transcriptional, respectively, while the a4 common allele produced only a 2-fold enhancement. A similar pattern of enhancement was seen in K562 with a1, a2 and a4, however, the enhancement was higher in the untreated cells compared with the PMA-treated. The a4 common allele consistently gave the lowest level of transcriptional enhancement, despite the fact that we controlled for the variations in VNTR length by adding the same molar quantities of the different luciferase constructs to the recipient cells.

CONCLUSIONS (Baldwin Laboratory)

First, our data indicate that gene expression directed through NF-κB binding sites can be controlled by a previously unknown mechanism (i.e., direct targeting of low levels of constitutively active [nuclear] NF-κB) without induced nuclear translocation. This is a fundamentally important observation with wide implications. Second, our data indicate that NF-κB is required for certain (possibly all) oncogenes to neoplastically transform cells. This is a broadly significant conclusion since the inhibition of NF-κB by known inhibitors may prove useful in cancer therapy. Third, we find that NF-κB activity protects cells against apoptosis. This particular finding may explain the requirement for NF-κB in transformation since protection against apoptosis is likely to be a critical component of oncogenesis. Additionally, resistance of tumors to radiation or chemotherapy may be based on the activation of NF-κB by these stimuli (thus blunting the therapeutic potential of these therapies). Finally, we now show that an unexpected form(s) of NF-κB is activated in human breast cancer. Each of these findings is fundamentally important to our understanding of cancer, and are likely to be essential for the development and progression of breast cancer. These data provide an important new foundation for pursuing our original aims and provide hope for developing a new generation of cancer therapies based on the inhibition of NF-κB.

CONCLUSIONS (Conway Laboratory)

First, the increased resolution of PCR-based allelotyping methods demonstrated that a cluster of alleles differing in length by one or a few repeats exists around each common allele mode, and that allele length distributions differed between African-American and white subjects. Second, H-ras allele length was found to be tightly linked with MVR sequence for each of the H-ras common alleles such that each allele possesses its own characteristic, distinguishing 5' MVR sequence. Rare alleles possessed disorganized 5' sequences, suggesting their derivation by recombinatory mechanisms. Third, using PCR-based allelotyping, the breast cancer risk associated with a rare H-ras allele was found to be substantially elevated in black breast cancer patients, but was not significantly increased in whites, although the rare allele of 59 repeats in length occurred more frequently in white breast cancer patients. This difference may be related to the variation in allele spectra between the two populations. Fourth, the combination of oligonucleotide competition, gel shift and supershift studies suggests that NF-κB/p52 and p50, p53, (and probably AP-1) proteins may bind the H-ras VNTR. Fifth, transient transfection experiments demonstrate that the Ha-ras VNTR possesses transcriptional regulatory function and the common alleles a1, a2 and a4 produce, in general, decreasing levels of transcriptional enhancement. The VNTR length-related effects on transcription are of particular interest in light of the recent studies on the transcriptional effects of the short versus long insulin VNTR alleles in diabetes susceptibility (Kennedy et al, 1995; Bennett et al, 1995). Because even the common H-ras VNTR alleles possess variability in enhancer function, distinguishing between "rare/high risk" and "common/low risk" alleles may be more complex than we initially thought. Although some confirmatory studies are still needed, the results of this work suggest that the H-ras VNTR modifies the transcription of a nearby gene (or genes), possibly the H-ras protooncogene. Interestingly, our NF-κB/p52 and p50 supershift findings are consistent with those of Dr. Baldwin's lab which demonstrated increased nuclear localization of these proteins in breast tumors.

MANUSCRIPTS ACCEPTED, IN PRESS OR IN PREPARATION:

C.-Y. Wang, M. Mayo, and A. Baldwin. (1996). TNF- and Cancer Therapy-Induced Apoptosis: Potentiation by Inhibition of NF-κB. *Science* 274, 784-787.

T. Finco, J. Westwick, J. Norris, A. Beg, C. Der and A. Baldwin. (1997). Oncogenic H-Ras-Induced Signaling Activates NF-κB Transcriptional Activity Which is Required for Cellular Transformation. *J. Biol. Chem.* 272, 24113-24116.

M. Mayo, C.-Y. Wang, P. Cogswell, K. Rogers-Graham, S. Lowe, C. Der and A. Baldwin. (1997). Requirement of NF-κB Activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* 278, 1812-1815.

P. Cogswell, D. Guttridge, and A. Baldwin. (1997). Selective activation of NF-κB subunits in human breast cancer: potential role for NF-κB2/p52 and Bcl-3. In review, *J. National Cancer Institute*.

Conway, K., Edmiston, S.N., Hulka, B.S., Garrett, P.A., Liu, E.T. (1996) Internal sequence variations in the Ha-ras variable number tandem repeat rare and common alleles identified by minisatellite variant repeat polymerase chain reaction. *Cancer Research* 56: 4773-4777.

Conway, K., Edmiston, S.N., Joens, S., Hulka, B.S., Garrett, P.A., Liu, E.T. Allelotyping of the Ha-ras variable number tandem repeat (VNTR) in a breast cancer case-control study. Submitted.

Conway, K. and Edmiston, S.N. Allele length effects of the H-ras VNTR on transcriptional regulation. In preparation.

The research for these manuscripts was directly supported by grant DAMD-94-J-4053.

MEETING ABSTRACTS:

A. Baldwin. A role for NF-κB in tumorigenesis and in protection against cancer therapies. The Department of Defense Breast Cancer Research Program Meeting. October 31 - November, 4, 1997. Page 565.

APPENDIX

1. Figures 1-3: below.

2. Papers attached: (Baldwin Laboratory)

Finco et al. (1997)
Wang et al. (1996)
Mayo et al. (1997)
Cogswell et al. (1997)

3. Papers attached: (Conway Laboratory)

Conway et al (1996).
Conway at al (submitted)

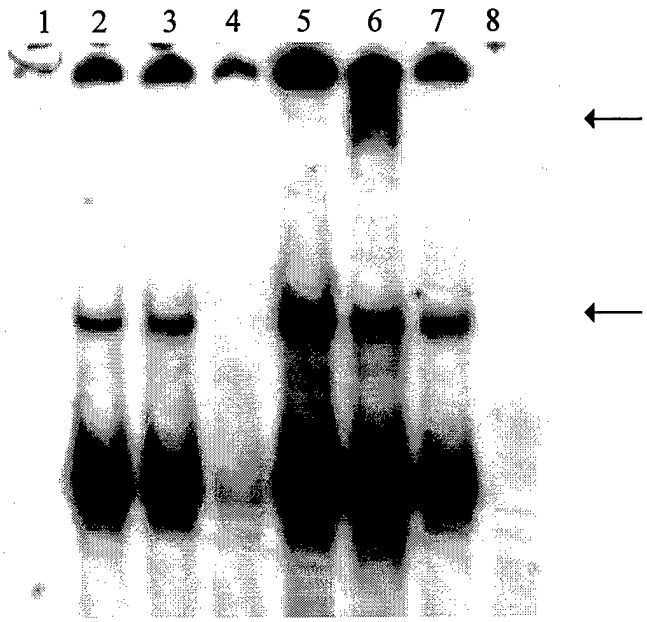


Figure 1: Supershift demonstrating NF-κB/p52 binding to a doublet of the H-ras VNTR repeat type 3 in the presence of NF-κB antisera. *Lane 1*; VNTR probe alone, *Lane 2*; nuclear extract from MCF-7, *Lane 3*; extract from TNF-α treated MCF-7 cells. Lanes 4-8 show H-ras probe plus TNF-α-induced MCF-7 extract and: *Lane 4*; competition by NF-κB sequence, *Lane 5*; NF-κB/p50 antisera showing enhanced binding to the VNTR (lower arrow), *Lane 6*; NF-κB/p52 antisera showing supershift (upper arrow), *Lane 7*; lack of competition by SRE sequence, *Lane 8*: competition by unlabelled H-ras VNTR type 3 repeat.

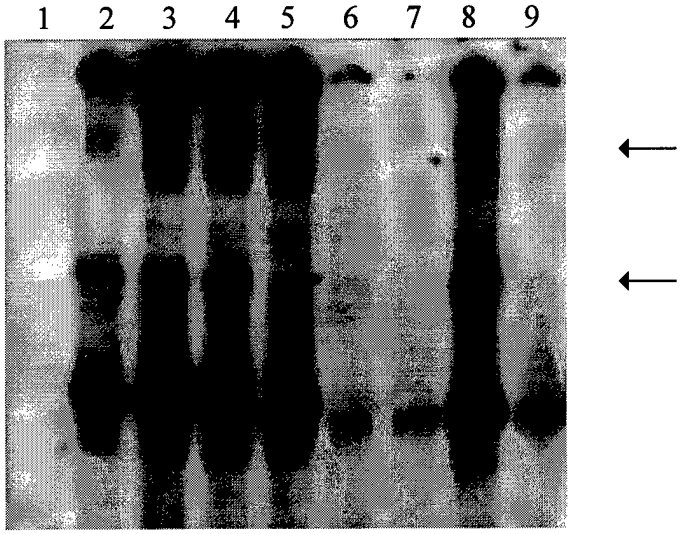


Figure 2: Enhanced p53 binding to a doublet of the H-ras VNTR repeat type 3 in the presence of p53 antisera *Lane 1*; VNTR probe alone, *Lane 2*; MCF-7 nuclear extract, Lanes 3-9 show the H-ras probe plus MCF-7 extract and: *Lanes 3 and 4*; p53 antibodies Ab1 and Ab6, respectively, showing enhanced binding (upper arrow), *Lane 5*; both p53 antibodies Ab1+Ab6 showing enhanced binding (upper arrow) and loss of binding in lower band (lower arrow), *Lane 6*; competition by p53 sequence, *Lane 7*; competition by unlabeled H-ras VNTR type 3 repeat, *Lane 8*; lack of competition by SRE sequence, *Lane 9*; competition by NF-κB sequence.

REFERENCES:

Baldwin, A. (1996). The NF-kB and IkB proteins: new discoveries and insights. Annual Rev. of Immunology 14: 649-681.

Bartek, J., Bartkova, J., Vojtesek, B., et al. Patterns of expression of the p53 tumor suppressor in human breast tissues in-situ and in-vitro. Int. J. Cancer 46: 839-844 (1990).

Beg, A. and A. Baldwin. (1993). The IkB proteins: multifunctional regulators of Rel/NF-kB transcription factors. Genes and Dev. 7: 2064-2070.

Bennett, S., A. Lucassen, S. Gough, E. Powell, D. Undlien, L. Pritchard, M. Merriman, Y. Kawaguchi, M. Dronsfield, F. Pociot, J. Nerup, N. Bouzekri, A. Cambon-Thomsen, K. Ronningen, A. Barrett, S. Bain and J. Todd. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus (1995). Nature Genetics 9: 284-292.

Conway, K., Edmiston, S.N., Hulka, B.S., Garrett, P.A., Liu, E.T. Internal sequence variations in the Ha-ras VNTR rare and common alleles identified by minisatellite variant repeat (MVR)-polymerase chain reaction (PCR). Cancer Res. 56: 4773-4777 (1996).

Finco, T. and A. Baldwin. (1995). Mechanistic aspects of NF-kB regulation: the emerging role for phosphorylation and proteolysis. Immunity, 3: 263-272.

Fisher, D. (1994). Apoptosis in cancer therapy: crossing the threshold. Cell 78: 539-542.

Friend, S. p53: a glimpse at the puppet behind the shadow play. Science 265: 334-335 (1994).

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., Shay, J.W. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol. 12: 2866-2871 (1992).

Futreal, P., Q. Liu, D. Shattuck-Eidens et al. (1994). BRCA1 mutations in primary breast and ovarian carcinomas. Science 266: 120-122.

Galang, C., J. Garcia-Ramirez, P. Solski, J. Westwick, C. Der, N. Neznanov, R. Oshima, and C. Hauser (1996). Oncogenic Neu/HER2 increases ets, Ap-1 and NF-kB dependent gene expression and inhibiting ets activation blocks neu-mediated cellular transformation. J. Biol. Chem. 271: 7992-7998.

Garrett, P.A., Hulka, B.S., Kim, Y.L., Farber, R.A. HRAS protooncogene polymorphism and breast cancer. Cancer Epidemiology, Biomarkers and Prevention 2: 131-138 (1993).

Green, M. and Krontiris, T.G. Allelic variation of reporter gene activation by the HRAS1 minisatellite. Genomics 17: 429-434 (1993).

Gualberto, A. and A. Baldwin. (1995). p53 and Sp1 interact and cooperate in the TNF-induced transcriptional activation of the HIV LTR. J. Biol. Chem. 270: in press.

Hupp, T.R., Meek, D.W., Midgley, C.A., Lane, D.P. Regulation of the specific DNA binding function of p53. Cell 71: 875-886 (1992).

Kennedy, G., M. German and W. Rutter. The minisatellite locus in the diabetes suspectibility locus IDDM2 regulates insulin transcription (1995). Nature Genetics 9: 293-298.

Krontiris, T., B. Devlin, D. Karp, N. Robert and N. Risch. An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. New Engl. J. Med. (1993) 329: 517-523.

Lippman, M. and R. Dickson. (1989). Mechanisms of normal and malignant breast epithelial growth regulation. J. Steroid Biochem. 34: 107-121.

Olson, D. and A. Levine. (1994). The properties of p53 proteins selected for the loss of suppression of transformation. Cell Growth Differ. 5: 61-71.

Rotter, V., O. Foord and N. Navot. 1993. In search of the functions of normal p53 protein. Trends Cell Biol. 3: 46-49.

Saitoh, S., J. Cunningham, E. De Vries, R. McGovern, J. Schroeder, A. Hartmann, H. Blaszyk, L. Wold, D. Schaid, S. Sommer and J. Kovach. p53 gene mutations in breast cancers in midwestern US women: null as well as missense mutations are associated with poor prognosis (1994). Oncogene 9: 2869-2875.

Seth et al. p53 mutations in women with breast cancer and a previous history of benign breast disease. European J. Cancer 6: 808-812 (1990).

Trepicchio, W.L., Krontiris, T.G. Members of the rel/NF-kB family of transcriptional regulatory proteins bind the HRAS1 minisatellite DNA sequence. Nucl. Acids Res. 20: 2427-2434 (1992).

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side.)

Form Approved
OMB No. 0704-0237
Expires Jun 30, 1992

1a. NAME OF CONTRACTOR / SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
Albert S. Baldwin	DAMD-17-94-J-4053			a. INTERIM [ ] b. FINAL [X]
b. ADDRESS (Include ZIP Code) Lineberger Cancer Center UNC, Chapel Hill, NC 27599	d. AWARD DATE (YYMMDD) July 1, 1994	b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYMMDD)	4. REPORTING PERIOD (YYMMDD) a. FROM 94-07-01 b. TO 98-06-30

SECTION I – SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR / SUBCONTRACTOR (If "None," so state) NONE

a. NAME(S) OF INVENTOR(S) (Last, First, MI)	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS (1) United States (a) Yes	(b) No	(2) Foreign (a) Yes	(b) No	e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (1) Yes	(2) No

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR / SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) Name of Inventor (Last, First, MI)	(2) (a) Name of Inventor (Last, First, MI)	(1) Title of Invention	(2) Foreign Countries of Patent Application
(b) Name of Employer	(b) Name of Employer		
(c) Address of Employer (Include ZIP Code)	(c) Address of Employer (Include ZIP Code)		

SECTION II – SUBCONTRACTS (Containing a "Patent Rights" clause)

6. SUBCONTRACTS AWARDED BY CONTRACTOR / SUBCONTRACTOR (If "None," so state) NONE

a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS (Include ZIP Code)	c. SUBCONTRACT NO.(S)	d. DFAR "PATENT RIGHTS" (1) Clause Number	(2) Date (YYMM)	e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES (YYMMDD) (1) Award	(2) Estimated Completion

SECTION III – CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR / SUBCONTRACTOR (Not required if [ ] Small Business or [X] Non-Profit organization.) (X appropriate box)

a. NAME OF AUTHORIZED CONTRACTOR / SUBCONTRACTOR OFFICIAL (Last, First, MI)

b. TITLE

c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

d. SIGNATURE Albert S Baldwin

e. DATE SIGNED 9/11/98

DD Form 882, OCT 89 *Previous editions are obsolete.* 971284

SALARIED PERSONNEL

Name	Start Date - End Date
Ashburner, Brian P.	9/18/95 – 7/31/96
Baldwin, Jr., Albert S.	7/01/94 – 6/30/95 7/01/95 – 8/31/96 9/01/96 – 6/30/97 7/01/97 – 9/30/97
Dorsey, Kathleen	7/01/97 – 12/31/97 7/01/94 – 8/31/94 3/16/95 – 6/30/95 7/01/95 – 8/31/96 9/01/94 – 9/29/94
Edmiston, Sharon N.	10/13/97 – 5/24/98 9/30/95 – 4/14/96 3/07/95 – 6/30/95 7/01/94 – 1/23/95 7/01/95 – 9/29/95 8/01/97 – 9/28/97 4/15/96 – 8/04/96 9/29/97 – 10/12/97 8/05/96 – 8/31/96 9/01/96 – 6/30/97 7/01/97 – 7/31/97
Makarov, Sergei S.	7/01/94 – 8/31/94
McMaster, Mary L.	1/01/95 – 8/31/95

Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 39, Issue of September 26, pp. 24113–24116, 1997

Oncogenic Ha-Ras-induced Signaling Activates NF-κB Transcriptional Activity, Which Is Required for Cellular Transformation*

(Received for publication, June 16, 1997, and in revised form, July 22, 1997)

Timothy S. Finco‡§¶, John K. Westwick‡‖, Jacqueline L. Norris‡ ‡‡, Amer A. Beg§§, Channing J. Der‡§‖, and Albert S. Baldwin, Jr.‡§¶¶‖‖**

From the ‡Lineberger Comprehensive Cancer Center, §Curriculum in Genetics and Molecular Biology, ‖Department of Pharmacology, and ¶¶Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-7295 and the §§Department of Biological Sciences, Columbia University, New York, New York 10027

Ras proteins function in stimulating cell proliferation and differentiation through the activation of Raf-dependent and Raf-independent signal transduction pathways and the subsequent activation of specific transcription factors. The transcription factor NF-κB has been widely studied as a regulator of genes involved in immune and inflammatory responses. A variety of stimuli activate NF-κB through the induced phosphorylation and degradation of the inhibitor IκB followed by nuclear translocation of NF-κB. We show here that oncogenic forms of Ha-Ras activate NF-κB, not through induced nuclear translocation, but rather through the activation of the transcriptional function of the NF-κB RelA/p65 subunit. Importantly, RelA/p65 −/− cells are inefficient in the activation of κB-dependent gene expression in response to oncogenic Ras expression. Furthermore, IκBα expression blocks focus formation in NIH3T3 cells induced by oncogenic Ras. These results demonstrate that NF-κB is a critical downstream mediator of Ha-Ras signaling and oncogenic potential.

Members of the Ras family of GTP-binding proteins serve as essential mediators in the ability of a variety of extracellular stimuli to regulate cellular proliferation and differentiation (1, 2). Oncogenic mutations in *ras* alleles, which occur in approximately 30% of human cancers, lead to chronic GTP binding, which initiates the activation of signal transduction cascades. In this regard, Ras is known to stimulate both the Raf/MEK[1]/ERK pathway as well as the MEKK/SEK/JNK pathway (3–7). Activation of these and other protein kinase cascades (8–10) is critical for the ability of Ras to exert both its normal and oncogenic functions. The ultimate targets of the Ras-induced signal transduction pathways are transcription factors (see Ref. 4), which regulate the expression of genes involved in proliferation and oncogenesis. Two transcription factors, Ets and c-Jun, have been shown to be essential for Ras-induced gene expression and for Ras-mediated cell transformation *in vitro* and tumorigenesis (11, 12). In these cases, Ras-induced signaling pathways activate the transcriptional function of both Ets and c-Jun via induced phosphorylation of their transcriptional activation domains (Ref. 13 and reviewed in Ref. 4).

The NF-κB family of proteins has been studied largely for the ability of these transcription factors to regulate a variety of genes involved in immune and inflammatory responses (reviewed in Ref. 14). The activation of these genes in response to inflammatory cytokines, T cell activation signals, lipopolysaccharide, etc. involves the targeted phosphorylation and degradation of the NF-κB inhibitor IκB, allowing nuclear translocation of NF-κB (reviewed in Ref. 14). Additionally, growing evidence indicates that NF-κB may play an important role in controlling cellular proliferation. For example, the c-*myc* proto-oncogene has been shown to be transcriptionally regulated by NF-κB (15), and antisense inhibition of IκBα leads to cellular transformation of NIH3T3 cells (16). Furthermore, members of the NF-κB and IκB families are associated with chromosomal translocations found in certain lymphomas (for example, see Ref. 17).

We and others previously demonstrated that transient transfection of oncogenic forms of Ha-Ras or of Raf-1 leads to the activation of reporter gene expression controlled by multiple NF-κB sites (18, 19). Consistent with the previous co-transfection studies, κB-dependent gene expression was elevated significantly in both Ras- and Raf-transformed cells as compared with the parental 3T3 cells. Interestingly, increased NF-κB binding activity was not detected in the Ras- or Raf-transformed cells. However, the activity of the transcriptional activation domain of the NF-κB RelA/p65 subunit was significantly increased in these cells. p65 −/− fibroblasts exhibited a reduced κB-dependent transcription response to either oncogenic Ras or Raf but retained their ability to activate the p65/RelA transcriptional activation domain. Finally, oncogenic Ras focus-forming activity was blocked by IκBα expression. These data indicate that NF-κB is an important downstream target for Ras-activated signal transduction pathways.

EXPERIMENTAL PROCEDURES

Cells and Transfections—NIH3T3 cells, the Ha-Ras and Raf-1-transformed counterparts, and the p65 +/− and p65 −/− mouse embryo fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin, and streptomycin. DNA transfections were performed by the calcium phosphate precipitation

* This research was supported by National Institutes of Health Grants CA72771 (to A. S. B.) and CA69577 (to C. J. D.) and by Department of the Army Grant DAMD-94-J-405 (to A. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: Howard Hughes Medical Inst., University of California, San Francisco, CA 94143.

** Lineberger Cancer Center Fellow during the course of this work. Present address: Signal Pharmaceuticals, San Diego, CA 92121.

‡‡ Supported by a National Institutes of Health postdoctoral fellowship.

‖‖ To whom correspondence should be addressed: Lineberger Comprehensive Cancer Center, CB# 7295, University of North Carolina School of Medicine, Chapel Hill, NC 27599. Tel.: 919-966-3652; Fax: 919-966-0444.

[1] The abbreviations used are: MEK, MAP/ERK kinase; ERK, extracellular signal-regulated kinase; JNK, Jun kinase; CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; LTR, long terminal repeat; DHFR, dihydrofolate reductase; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus.

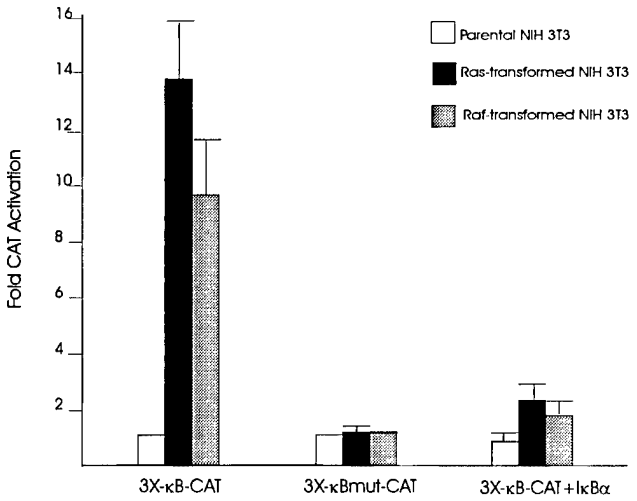


FIG. 1. **κB-dependent gene expression is increased in Ras- as well as Raf-transformed cells.** Either the κB-dependent CAT reporter (3X-κB-CAT) or its mutant version (3X-κBmut-CAT) was transfected into Ras-transformed NIH3T3 cells, Raf-transformed NIH3T3 cells, or parental NIH3T3 cells either alone or with the CMV-IκBα expression vector. CAT activity was measured as described under "Experimental Procedures." Data are presented as mean ± S.D.

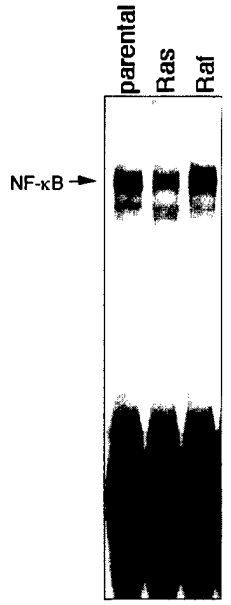


FIG. 2. **NF-κB binding activity is not increased in Ras- or Raf-transformed NIH3T3 cells.** Gel mobility shift assays were performed on extracts of double sucrose pad-purified nuclei from parental NIH3T3 cells or the Ras- and Raf-transformed counterparts as described under "Experimental Procedures." NF-κB is shown by the *arrow*.

method as described previously (18). The plasmid pGEM or salmon sperm DNA was used to equalize the amount of DNA transfected in each experiment to 15 μg. CAT analysis and luciferase assays were performed as previously reported (18, 20). In all cases, 1 unit of relative activity represents the CAT or luciferase activity obtained after transfection of the reporter gene alone. All experiments were performed at least three times with similar results.

Plasmids—The following plasmids have been described previously: activated Raf (RafBXB) and activated Ras (v-Ha-Ras) expression vectors (18), the IκBα expression vector (21), the super-repressor IκBα expression vector (22), the expression vector encoding the Gal4 DNA-binding domain fused to the C-terminal domain of p65/RelA (Gal4p65aa519–551 (23)), the reporters 3X-κB-CAT and 3X-mutκB-CAT (18), the HIV LTR-CAT and HIV-ΔκB-CAT reporters (18), 5X-Gal4-CAT (24), and DHFR-CAT (25).

Extracts and Gel Mobility Shift Assays—Nuclear and cytoplasmic extracts were prepared as described previously (26). For double sucrose pad purification, washed nuclei were resuspended in lysis buffer lacking Nonidet P-40 and layered on a sucrose pad (30% sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes, 2 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, and 1 mM dithiothreitol) and centrifuged for 15 min at 3,000 rpm in an HB4 rotor. The sucrose pad was then removed, the nuclei resuspended, and the process repeated. Nuclear extracts were then prepared from the sucrose pad-purified nuclei. Gel mobility shift assays (EMSAs) were performed as described previously (26).

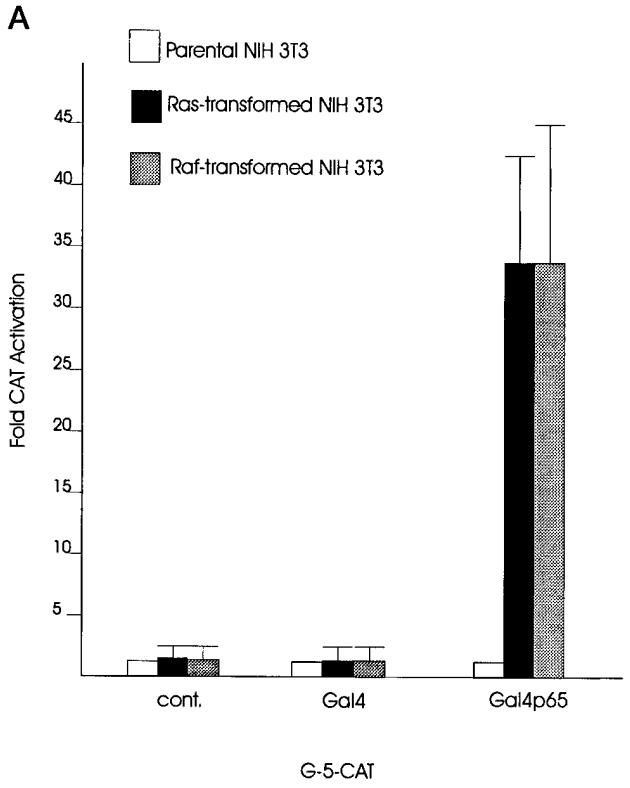
Focus Formation Assays—NIH3T3 cells were transfected by calcium phosphate coprecipitation essentially as described (27). For each 60-mm plate, 10 ng of Ras expression vector pZip-*ras*H(61L) was transfected, along with the indicated quantity of IκBα expression vector or empty vector. In all cases, the expression vector was normalized with the cognate empty expression vectors. Cells were fed every 2 days, and the appearance of foci of transformed cells was counted 14 days after transfection. Four plates per condition were transfected, and graphs represent the mean ± S.E. of these counts. Data are representative of four independent experiments performed in quadruplicate.

RESULTS

Oncogenic Ras- or Raf-transformed Cells Exhibit Increased κB-dependent Transcription without Increased Nuclear Accumulation of NF-κB—Previous transient cotransfection experiments indicated that expression of either oncogenic Ras or oncogenic Raf led to a significant activation of expression of a κB-dependent reporter (18). Consistent with the previous cotransfection data, the activity of a κB-dependent reporter was significantly elevated in both Ras- and Raf-transformed cells but not in the parental NIH3T3 cells (Fig. 1*A*). A reporter mutated in the NF-κB sites did not exhibit this enhanced activity, and expression of the NF-κB inhibitor IκBα blocked the Ras- and Raf-induced activation of κB-dependent reporter activity, indicating that NF-κB regulates the transcription response. A similar result was obtained with the NF-κB-dependent HIV-LTR reporter (data not shown). Additionally, expression of the non-Ras-responsive DHFR-CAT reporter was approximately equivalent in each of the three cell types (data not shown) showing that the differential responses observed in the transformed cells were not due to differential uptake of plasmids.

To determine if oncogenic Ras as well as oncogenic Raf activated nuclear accumulation of NF-κB, gel mobility shift assays (EMSAs) were performed with nuclear extracts from parental NIH3T3 cells, oncogenic Ras-transformed NIH3T3 cells, or Raf-transformed NIH3T3 cells. To demonstrate that binding activity was exclusively nuclear, extracts were prepared from double sucrose pad-purified nuclei. Immunoblotting for NF-κB1/p105 (which is cytoplasmic) indicated that there was no cytoplasmic contamination in the nuclear preparations (data not shown). As shown in Fig. 2, NF-κB was detected in the nuclei of each of the different cells at similar levels. Antibody "supershift" experiments showed that this binding activity is authentic, p65/RelA-containing NF-κB (data not shown). These results were surprising and indicated that the activation of κB-dependent transcription observed in the transfection experiments shown in Fig. 1 was not controlled by the induced nuclear accumulation of NF-κB but suggested that this response was mediated by the relatively low levels of constitutively nuclear NF-κB in NIH3T3 fibroblasts. It should be noted that transient transfection of oncogenic Ras into 3T3 cells or the induction of oncogenic Ras in Rat-1 cells led to an approximate 3-fold increase in nuclear NF-κB (data not shown); however established Ras-transformed cells did not exhibit this property. These experiments indicated that oncogenic Ras or Raf can activate κB-dependent transcription without enhancing nuclear levels of NF-κB.

Oncogenic Forms of Ras and Raf Activate the Transcriptional Function of NF-κB p65/RelA—To explain the activation of κB-dependent transcription by oncogenic forms of Ras or Raf without an induction of NF-κB nuclear translocation, we asked whether the transcriptional activation function of NF-κB was stimulated



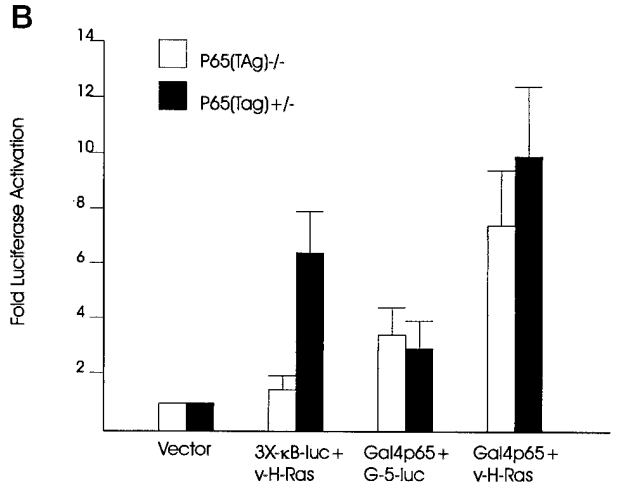


FIG. 3. **The p65/RelA subunit of NF-κB is functionally activated by Ras and is required for Ras to efficiently activate κB-dependent gene expression.** *A*, either the vector (Gal4p65) encoding a fusion protein between the DNA binding of Gal4 and the TA1 transcriptional activation domain of p65/RelA or the Gal4 vector was transfected into NIH3T3 cells or the Ras- or Raf-transformed counterparts of these cells. CAT activity was determined as described under "Experimental Procedures." *B*, SV40 large T (Tag) immortalized embryonic fibroblasts isolated from p65 −/− or p65 +/− mice were transfected with the κB-dependent CAT reporter alone with oncogenic Ras or with the Gal4p65 vector alone or with oncogenic Ras. CAT activity was measured as described and is presented as mean ± S.D.

in the transformed cells. A plasmid (Gal4p65) encoding a fusion of the C-terminal (TA1) transactivation domain of RelA (23) with the DNA-binding domain of the yeast transcription factor Gal4 was transfected into parental NIH3T3 cells, or Ras- or Raf-transformed cells, along with a luciferase reporter containing upstream Gal4-binding sites. Luciferase activity driven by Gal4p65 or Gal4 was compared in the three cell types. The results indicate that the Gal4p65 construct is strongly active in the Ras- and the Raf-transformed cells but only weakly active in

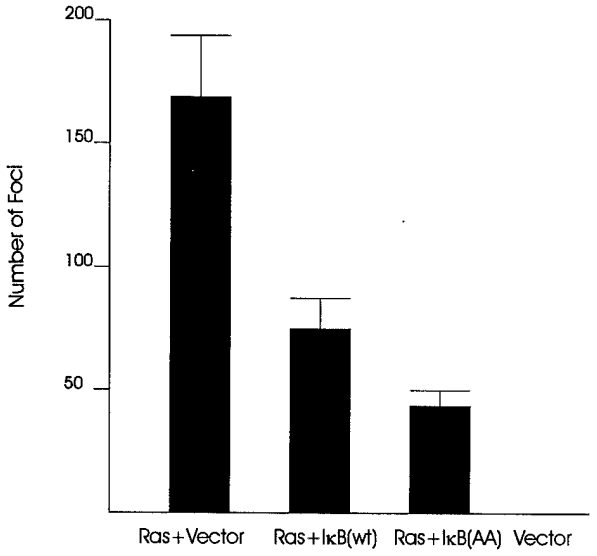


FIG. 4. **IκBα blocks focus formation induced by oncogenic Ras.** NIH3T3 cells were transfected with the oncogenic Ras expression vector (plus empty CMV vector) or with the empty vector Ras expression vector. Alternatively, the Ras expression vector was co-transfected with a vector encoding the wild-type form of IκBα or a vector encoding the modified, super-repressor form of IκBα (*IκBα(AA)*) as described under "Experimental Procedures." Results are presented as foci per plate and are the mean ± S.D.

the untransformed 3T3 cells (Fig. 3*A*). EMSA experiments indicated that there was not an increase in the DNA binding activity of the Gal4-p65 protein in the Ras- and Raf-transformed cells (data not shown). These results demonstrate that oncogenic Ras or Raf activates a signal transduction pathway that stimulates p65/RelA transcriptional activation function controlled by the TA1 transcriptional activation domain.

The results described above suggested that the RelA subunit of NF-κB may function as a critical downstream transcriptional effector for the Ras oncoprotein. To test this hypothesis, we utilized immortalized RelA +/− and RelA −/− embryonic fibroblasts (28) for transfection and gene expression studies. Oncogenic Ras was ineffective at activating κB-dependent gene expression in the p65 −/− cells (approximately a 2-fold activation), whereas effective Ras activation of κB-dependent gene expression (approximately 7-fold) was observed in the RelA +/− cells, as expected (Fig. 3*B*). To show that the Ras-responsive signal transduction pathway was still operative in the RelA −/− cells, the Gal4p65 construct was cotransfected with either activated Ha-Ras or activated Raf-1. Ras activated the Gal4p65 construct as effectively in RelA +/− cells as in RelA −/− cells. These results demonstrate that the RelA/p65 subunit of NF-κB is required for oncogenic Ras to effectively activate gene expression driven by consensus NF-κB-binding sites.

NF-κB Is Required for Ras-mediated Cellular Transformation—To determine whether NF-κB is required for cellular transformation controlled by oncogenic Ha-Ras, we determined whether the inhibition of NF-κB would affect the ability of Ras to cause formation of transformed foci in cultured NIH3T3 cells. To specifically inhibit NF-κB activity, we used an expression vector encoding IκBα, which can enter the nucleus and relocate NF-κB to the cytoplasm (29). Transfection of pZIP-*ras*(61L) together with the empty CMV vector yielded an average of approximately 160 foci/plate (Fig. 4). Co-expression of oncogenic Ras with wild-type IκBα blocked focus formation activity by greater than 50%. Co-expression with a super-repressor form of IκBα (mutated in serines 32 and 36) that is unable to be inducibly phosphorylated or degraded in response to stimuli (see Ref. 22) blocked focus formation by approxi-

mately 70–75% (Fig. 4). Expression of IκBα did not block expression of the promoter driving Ras expression or Ras protein expression (data not shown). Interestingly, IκBα was unable to block the ability of activated Rho (Rho63L) to induce focus formation. In these experiments, activated Rho yielded approximately 20 foci/plate, and IκBα expression did not reduce this number of foci (data not shown).

DISCUSSION

The data presented here indicate that oncogenic *ras* alleles activate NF-κB-dependent transcription, not through the induced nuclear translocation of NF-κB, but rather through the stimulation of the transcriptional activation function of NF-κB via the targeting of the RelA/p65 subunit. Furthermore, the data indicate that NF-κB is required for Ras to initiate efficient cellular transformation and that NF-κB plays a role in mediating certain essential aspects of cellular transformation. Thus, NF-κB joins Ets family members (13) and c-Jun (4, 12) as downstream targets of oncogenic Ras that are required for Ras-mediated cellular transformation.

How does Ras activate NF-κB functional activity? Our data strongly indicate that the transcriptional activation function of RelA/p65 NF-kB is potentiated in both Ras- as well as Raf-transformed cells, and at least two mechanisms exist to explain this phenomenon. First, a Ras-initiated signal transduction pathway may target the p65 transcriptional activation domain for phosphorylation, which may allow enhanced interactions with a transcriptional co-activator or with basal transcriptional machinery. Such a mechanism appears to be operative for both Ets-1 and -2 and for c-Jun (4, 13). A second mechanism may be that a transcriptional co-activator is modified such that it interacts functionally with p65 transcriptional activation domain. Also of importance is identification of the signal transduction pathway that is initiated by Ras to stimulate NF-κB transcriptional activity. Since both oncogenic Ras as well as oncogenic Raf stimulate κB-dependent activity, it may be assumed that the relevant pathway is downstream of Raf and is, therefore, the MEK/ERK pathway. However, inhibitors of this pathway did not block the ability of Ras to activate κB-dependent transcription, and dominant negative forms of kinases in the SEK/JNK pathway were able to block this response.[2] Thus the ability of Raf to activate κB-dependent gene expression in a MEK/ERK-independent pathway may be explained by the recent observation that Raf stimulates JNK activity via an autocrine mechanism (30).

Prior studies have shown that the major regulatory mechanism involved in regulating κB-dependent transcription is induced nuclear translocation (see Ref. 14). Our data indicate that significant κB-dependent transcription can be realized without enhancing the constitutive, low nuclear levels of NF-κB. This suggests that under some circumstances the functional activity of NF-κB can be separated from induction of nuclear translocation. Consistent with this concept are the recent observations that the tyrosine kinase inhibitor genistein blocks the ability of NF-κB to stimulate transcription of an NF-κB-dependent reporter but is not able to block nuclear translocation of NF-κB (31) and that phorbol 12-myristate 13-acetate can activate the TA2 transcriptional activation domain of RelA/p65 (23).

Evidence that NF-κB is required for Ras-mediated cellular transformation is consistent with several observations indicating a role for NF-κB in controlling cell growth. First, it has been shown that NF-κB can regulate c-*myc* gene expression. Second, antisense studies indicate that NF-κB can control oncogenesis. These experiments utilized antisense to p65 to block oncogene-controlled transformation (32, 33) and antisense to IκBα to induce transformation of NIH3T3 cells (16). Additionally, other oncogenes such as Her2/NEU are known to activate NF-κB (34). Thus, the activation of NF-κB may be common to a number of oncogenes, particularly those that utilize Ras-controlled signaling pathways. Additionally, we have been able to show NF-κB activation is required to block a Ras-induced apoptotic response.[3] This result is consistent with recent data (22, 35–38) that NF-κB activation can block the induction of apoptosis. Further experiments are required to establish the exact role that NF-κB plays in controlling Ras-mediated oncogenesis.

Acknowledgment—We gratefully acknowledge Dr. P. Baeuerle for the kind gift of the Gal4p65 construct.

REFERENCES

1. Boguski, M. S., and McCormick, F. (1993) *Nature* **366,** 643–654
2. Khosravi-Far, R., and Der, C. J. (1994) *Cancer Metastasis Rev.* **13,** 67–89
3. Marshall, C. J. (1995) *Cell* **80,** 179–195
4. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
5. Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. (1994) *Cell* **76,** 1025–1037
6. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R., Johnson, G., and Karin, M. (1994) *Science* **266,** 1719–1723
7. Olsen, M., Ashworth, A., and Hall, A. (1995) *Science* **269,** 1270–1272
8. Rodriguez-Viciana, P., Warne, P., Khwaja, A., Marte, B., Pappin, D., Das, P., Waterfield, M., Ridley, A., and Downward, J. (1997) *Cell* **89,** 457–467
9. White, M., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M. (1995) *Cell* **80,** 533–541
10. Khosravi-Far, R., White, M., Westwick, J., Solski, P., Chrzanowska-Wodnicka, M., Van Aelst, L., Wigler, M., and Der, C. (1996) *Mol. Cell. Biol.* **16,** 3923–3933
11. Langer, S., Bortner, D., Roussel, M., Sherr, C., and Ostrowski, M. (1992) *Mol. Cell. Biol.* **12,** 5355–5362
12. Johnson, R., Spiegelman, B., Hanahan, D., and Wisdom, R. (1996) *Mol. Cell. Biol.* **16,** 4504–4511
13. Yang, B.-S., Hauser, C., Henkel, G., Colman, M., Van Beveren, C., Stacey, K., Hume, D., Maki, R., and Ostrowski, M. (1996) *Mol. Cell. Biol.* **16,** 538–547
14. Baldwin, A. (1996) *Annu. Rev. Immunol.* **14,** 649–681
15. La Rosa, F., Pierce, J., and Sonenshein, G. (1994) *Mol. Cell. Biol.* **14,** 1039–1044
16. Beauparlant, P., Kwan, I., Bitar, R., Chou, P., Koromilas, A., Sonenberg, N., and Hiscott, J. (1994) *Oncogene* **9,** 3189–3197
17. Zhang, J., Chang, C., Lombardi, L., and Dalla-Favera, R. (1994) *Oncogene* **9,** 1931–1937
18. Finco, T., and Baldwin, A. (1993) *J. Biol. Chem.* **268,** 17676–17679
19. Galang, C., Der, C., and Hauser, C. (1994) *Oncogene* **9,** 2913–2921
20. Cogswell, P., Mayo, M., and Baldwin, A. (1997) *J. Exp. Med.* **185,** 491–497
21. Beg, A., Ruben, S., Scheinman, R., Haskill, S., Rosen, C., and Baldwin, A. (1992) *Genes Dev.* **6,** 1899–1913
22. Wang, C.-Y., Mayo, M., and Baldwin, A. (1996) *Science* **274,** 784–787
23. Schmitz, M., dos Santos Silva, M., and Baeuerle, P. (1995) *J. Biol. Chem.* **270,** 15576–15584
24. Blair, W., Bogerd, H., Madore, S., and Cullen, B. (1994) *Mol. Cell. Biol.* **14,** 7226–7234
25. Wade, M., Blake, M., Jambou, R., Helin, K., Harlow, E., and Azizkhan, J. (1995) *J. Biol. Chem.* **270,** 9783–9791
26. Finco, T., Beg, A., and Baldwin, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11884–11888
27. Clark, G., Westwick, J., and Der, C. (1997) *J. Biol. Chem.* **272,** 1677–1681
28. Beg, A., Sha, W., Bronson, R., Ghosh, S., and Baltimore, D. (1995) *Nature* **376,** 167–170
29. Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M., Bachelerie, F., Thomas, D., and Hay, R. (1995) *Mol. Cell. Biol.* **15,** 2689–2696
30. McCarthy, S., Samuels, M., Pritchard, C., Abraham, J., and McMahon, M. (1995) *Genes Dev.* **9,** 1953–1964
31. Yoza, B., Hu, J., and McCall, C. (1996) *J. Biol. Chem.* **271,** 18306–18309
32. Kitajima, I., Shinohara, T., Bilakovics, J., Brown, D., Xu, X., and Nerenberg, M. (1992) *Science* **258,** 1792–1795
33. Higgins, K., Perez, J., Coleman, T., Dorshkind, K., McComas, W., Sarmiento, U., Rosen, C., and Narayanan, R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9901–9905
34. Galang, C., Garcia-Ramirez, J., Solski, P., Westwick, J., Der, C., Neznanov, N., Oshima, R., and Hauser, C. (1996) *J. Biol. Chem.* **271,** 7992–7998
35. Beg, A., and Baltimore, D. (1996) *Science* **274,** 782–784
36. van Antwerp, D., Martin, S., Kafri, T., Green, D., and Verma, I. (1996) *Science* **274,** 787–789
37. Arsura, M., Wu, M., and Sonenshein, G. (1996) *Immunity* **5,** 31–40
38. Liu, Z., Hsu, H., Goeddel, D., and Karin, M. (1996) *Cell* **87,** 565

[2] J. Norris and A. S. Baldwin, Jr., unpublished observations.

[3] M. Mayo and A. S. Baldwin, Jr., submitted for publication.

Reprint Series
5 December 1997, Volume 278, pp. 1812–1815

SCIENCE

Requirement of NF-κB Activation to Suppress p53-Independent Apoptosis Induced by Oncogenic Ras

Marty W. Mayo, Cun-Yu Wang, Patricia C. Cogswell, Kelley S. Rogers-Graham, Scott W. Lowe, Channing J. Der, and Albert S. Baldwin Jr.*

Requirement of NF-κB Activation to Suppress p53-Independent Apoptosis Induced by Oncogenic Ras

Marty W. Mayo, Cun-Yu Wang, Patricia C. Cogswell, Kelley S. Rogers-Graham, Scott W. Lowe, Channing J. Der, Albert S. Baldwin Jr.*

The *ras* proto-oncogene is frequently mutated in human tumors and functions to chronically stimulate signal transduction cascades resulting in the synthesis or activation of specific transcription factors, including Ets, c-Myc, c-Jun, and nuclear factor kappa B (NF-κB). These Ras-responsive transcription factors are required for transformation, but the mechanisms by which these proteins facilitate oncogenesis have not been fully established. Oncogenic Ras was shown to initiate a p53-independent apoptotic response that was suppressed through the activation of NF-κB. These results provide an explanation for the requirement of NF-κB for Ras-mediated oncogenesis and provide evidence that Ras-transformed cells are susceptible to apoptosis even if they do not express the p53 tumor-suppressor gene product.

Mutations in a *ras* allele occur in 30% of all human tumors (*1*), making *ras* the most widely mutated human proto-oncogene. Both mitogen-activated protein (MAP) kinase–dependent and MAP kinase–independent pathways mediate Ras-induced cellular responses (*2*), and these signal transduction pathways ultimately control the activity of various transcription factors (*3*). The Ets, c-Myc, and c-Jun proteins are Ras-responsive transcription factors required for cellular transformation in vitro (*4*) and in vivo (*5*). The transcription factor NF-κB is also activated in response to oncogenic Ras (*6*), and this regulation occurs largely through the stimulation of the transcriptional function of the NF-κB RelA/p65 subunit (*7*). Moreover, NF-κB is required for Ras-mediated focus-forming activity (*7*), and activation of this transcription factor provides protection against apoptosis (*8*, *9*). Because NF-κB may play a direct role in cellular transformation (*10*) and because oncogenesis appears to require an anti-apoptotic function (*11*), we investigated whether oncogenic Ras requires NF-κB activation to block transformation-induced programmed cell death.

To determine whether the inhibition of NF-κB in Ras-transformed cells would initiate a cell death response, we used β-galactosidase (β-Gal) expression assays to measure cell viability. We inhibited NF-κB activity with a super-repressor form of IκBα (SR-IκBα), which cannot be phosphorylated (*12*) or degraded (*13*) and, therefore, blocks the nuclear translocation and subsequent transactivation of NF-κB –responsive genes (*8*). Parental NIH 3T3 and H-Ras–transformed cells (3T3 H-Ras[V12]) were cotransfected with a pCMV-LacZ reporter and with either an empty expression vector control or a vector encoding SR-IκBα. H-Ras–transformed NIH 3T3 cells expressing SR-IκBα displayed a decrease in the total number of β-Gal–positive cells as compared with cells transfected with the vector control (Fig. 1). In contrast, parental NIH 3T3

M. W. Mayo and P. C. Cogswell, Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA.
C.-Y. Wang, Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine; Curriculum in Genetics and Molecular Biology; and Department of Endodontics, University of North Carolina School of Dentistry, Chapel Hill, NC 27599, USA.
K. S. Rogers-Graham, Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA.
S. W. Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA.
C. J. Der, Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine; Curriculum in Genetics and Molecular Biology; and Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA.
A. S. Baldwin, Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine; Curriculum in Genetics and Molecular Biology; and Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA.

*To whom correspondence should be addressed at Lineberger Comprehensive Cancer Center, Campus Box 7295, University of North Carolina, Chapel Hill, NC 27599, USA.

cells expressing SR-IκBα showed no reduction in the number of β-Gal–positive cells, indicating that the expression from the pCMV-lacZ reporter was not affected by the super-repressor and that expression of IκBα did not kill nontransformed parental cells (Fig. 1). Similar results were obtained with H-Ras(V12)–transformed Rat-1 fibroblasts and C127 mouse mammary epithelial cells (*14*). Because gain-of-function mutations in either K- or N-*ras* are more prevalent in human cancers than are mutations in H-*ras* (*1*), we examined whether NF-κB served a similar protective role for other oncogenic Ras proteins. Like 3T3 H-Ras(V12) cells, NIH 3T3 cells transformed with other activated alleles of *ras* died after the expression of SR-IκBα (Fig. 1). Nontransformed NIH 3T3 cells stably expressing wild-type H-Ras did not exhibit a loss of cell viability in the presence of the SR-IκBα (*14*). These results indicate that oncogenic Ras (but not wild-type H-Ras) requires the cell survival function of NF-κB to overcome the activation of a death pathway initiated in transformed cells.

To determine whether the loss of cell viability was a direct effect of oncogenic Ras, we used the Rat-1:iRas cell line, which contains a stably integrated oncogenic H-*ras*(V12) gene under the controlled expression of an isopropyl-β-D-thiogalactopyranoside (IPTG)–inducible promoter (*15*). To inhibit NF-κB activation, we established the Rat-1:iRasI line, which constitutively expresses SR-IκBα. Moreover, a control line was established, Rat-1:iRasV, that contains the empty expression vector. The addition of IPTG induced similar amounts of $p21^{ras}$ protein in each of the cell lines, whereas only the Rat-1:iRasI cells displayed a larger immunoreactive band that corresponded to the Flag-tagged SR-IκBα (Fig. 2A). In contrast with the Rat-1:iRasV control cells, the Rat-1:iRasI line did not demonstrate increased nuclear NF-κB binding activity or increased transactivation of the 3x-κB–dependent reporter in response to

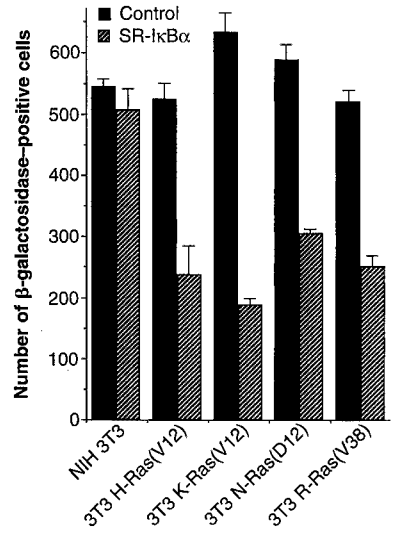


Fig. 1. Effects of SR-IκBα expression in parental and Ras-transformed NIH 3T3 cells. Parental NIH 3T3 and Ras-transformed NIH 3T3 cells were cotransfected (*8*) with pCMV-LacZ (pcDNA3-LacZ, Invitrogen; 0.2 μg per 24-well plate) and either an empty expression vector control (CMV-4) or with vector encoding SR-IκBα (1 μg per plate each). After 48 hours cells were fixed and stained with X-Gal, and β-Gal–positive cells were counted in each well. The results represent the mean ± SD of three independent experiments performed in triplicate.

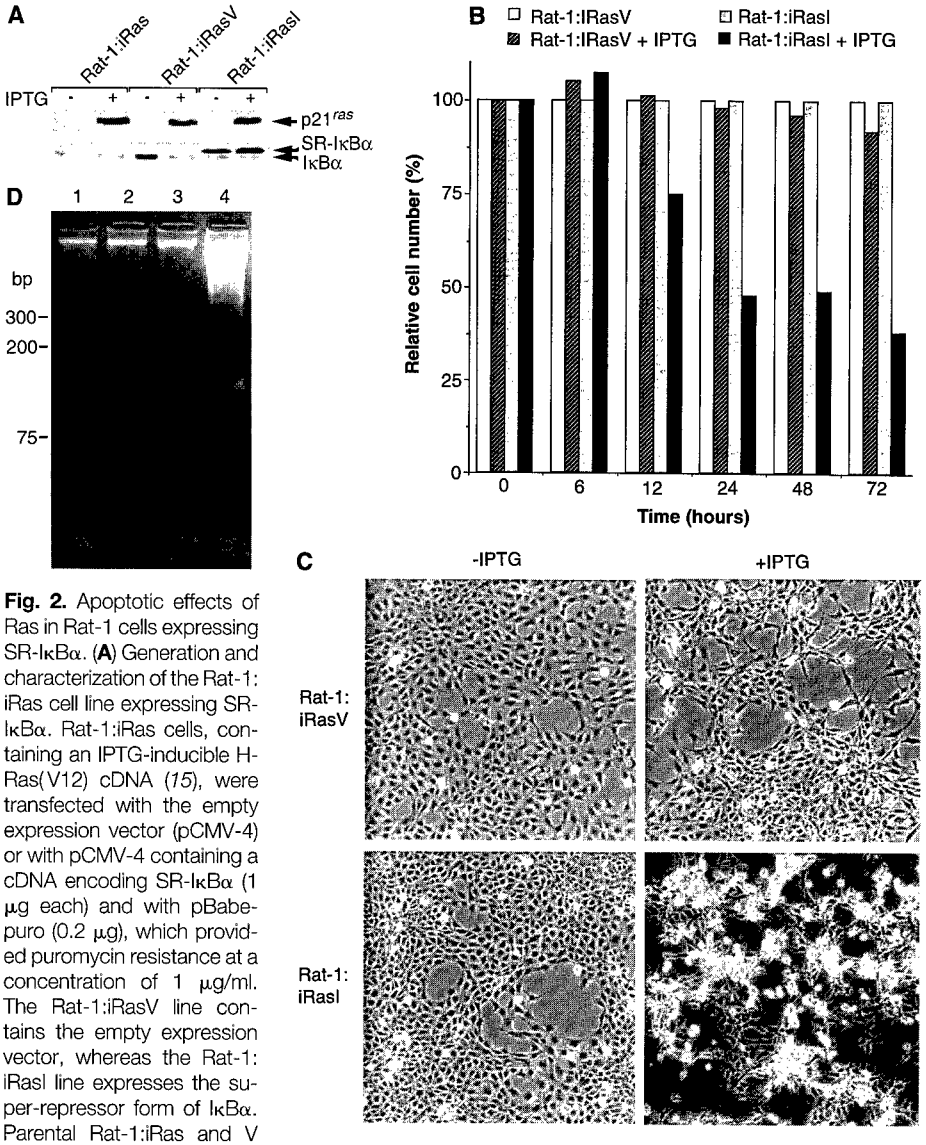


Fig. 2. Apoptotic effects of Ras in Rat-1 cells expressing SR-IκBα. (**A**) Generation and characterization of the Rat-1:iRas cell line expressing SR-IκBα. Rat-1:iRas cells, containing an IPTG-inducible H-Ras(V12) cDNA (*15*), were transfected with the empty expression vector (pCMV-4) or with pCMV-4 containing a cDNA encoding SR-IκBα (1 μg each) and with pBabe-puro (0.2 μg), which provided puromycin resistance at a concentration of 1 μg/ml. The Rat-1:iRasV line contains the empty expression vector, whereas the Rat-1:iRasI line expresses the super-repressor form of IκBα. Parental Rat-1:iRas and V and I cells were treated with 5 mM IPTG (Promega) for 24 hours, and protein expression was assessed by protein immunoblotting total cell extracts (60 μg per lane) with either a pan-Ras antibody (Ab-4, Calbiochem) or with an antibody specific for IκBα (Rockland). (**B**) Expression of oncogenic Ras and SR-IκBα. Rat-1:iRasI and Rat-1:iRasV cells (1×10^6) were plated into 100-mm dishes containing Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS), and 12 hours later cells were either left untreated or stimulated with 5 mM IPTG. Cells were harvested at the time indicated, and viable cells were identified by trypan blue dye exclusion. The data represent the mean (SD ≤ 2.7) of three independent experiments. (**C**) Apoptosis in Rat-1 cells expressing the SR-IκBα and H-Ras(V12). Micrographs (magnification, ×20) of Rat-1:iRasV and Rat-1:iRasI cells grown in DMEM containing 10% FBS in the absence or presence of IPTG for 36 hours. Dying, nonadherent cells are seen as refractile by phase contrast at this magnification. (**D**) DNA fragmentation in the absence of NF-κB activation. Rat-1:iRasI and Rat-1:iRasV cells were cultured at 1×10^5 cells/ml in DMEM containing 10% FBS in the absence or presence of IPTG. After 36 hours, cells were harvested, and genomic DNAs were isolated and resolved on 1.5% agarose gel. Representative DNAs include (lane 1) Rat-1:iRasV, (lane 2) Rat-1:iRasV + IPTG, (lane 3) Rat-1:iRasI, and (lane 4) Rat-1:iRasI + IPTG. Linear double-stranded DNA fragments were sized by means of a 1-kb DNA ladder (Gibco-BRL).

the expression of oncogenic Ras or tumor necrosis factor stimulation (*14*).

We used the Rat-1:iRasI cells to determine if the activation of Ras leads to a loss of cell viability under conditions where NF-κB is inhibited. Rat-1:iRasI and control cells were subjected to IPTG treatment, and total cell numbers were examined over a 3-day period. Greater than a 50% loss in cell viability was observed in the Rat-1:iRasI cells after Ras expression, as compared with the vector control cells (Fig. 2B). Similar results were obtained when five individual puromycin-resistant clones, expressing similar levels of the IκBα transgene, were pooled and analyzed (*14*). Both the Rat-1:iRasV and Rat-1:iRasI cells displayed distinct Ras-transformed morphology after the addition of IPTG (Fig. 2C) (*15*). Cell death induced by oncogenic Ras expression in Rat-1:iRasI cells bore the consistent hallmarks of apoptosis (*16*), including retraction of cellular processes, nuclear condensation, and a loss of adherence to the tissue culture dish (Fig. 2C). Induced expression of activated Ras in Rat-1:iRasI cells, but not the vector control cells, resulted in an increase in genomic DNA fragmentation evident 36 hours after the addition of IPTG, as detected by agarose gel electrophoresis (Fig. 2D). Furthermore, IPTG treatment induced apoptosis, as detected by the appearance of deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)–positive cells (*14*). Fluorescence-activated cell sorter (FACS) analysis of propidium iodide- and bromodeoxyuridine-stained cells confirmed that IPTG did not induce senescence (*17*) and that the cells that were <2N DNA represented apoptotic cells and not necrotic cells (*14*). Thus, the induction of oncogenic Ras expression in the presence of a specific inhibitor of NF-κB leads to enhanced apoptosis. Our results support the hypothesis that oncogenic Ras requires NF-κB activation to block transformation-induced programmed cell death.

The inability of activated Ras to transform primary cells is due, in part, to the expression of tumor-suppressor genes, such as *p53* (*18*) and *p16* (*19*). To determine if p53 had a role in the Ras-dependent killing, we examined whether $p53^{-/-}$ mouse embryo fibroblasts (MEFs) expressing oncogenic H-Ras(V12) required the cell survival function of NF-κB. We used $p53^{-/-}$ MEFs expressing either E1A, a viral oncogene product that activates NF-κB (*20*), or E1A plus H-Ras (*21*). The cells expressing E1A or E1A and H-Ras displayed enhanced NF-κB–dependent transcriptional activity, as compared with that in control $p53^{-/-}$ MEFs (*14*). Moreover, the total number of $p53^{-/-}$ MEFs expressing E1A or both E1A and H-Ras was reduced after the expression of SR-IκBα (Fig. 3A). In contrast, $p53^{-/-}$ MEFs showed little loss of viability after expression of the super-repressor form of IκB (Fig. 3A). Although E1A induces apoptosis in a p53-dependent manner (*22*), our data indicate that E1A can also induce apoptosis in a $p53^{-/-}$-deficient background if the protective function of NF-κB is inhibited. To confirm that E1A was not required for Ras-mediated killing, we transfected $p53^{-/-}$ MEFs with pCMV-LacZ, H-Ras(V12), and with either the empty expression vector or vector encoding SR-IκBα. $p53^{-/-}$ MEFs transfected with oncogenic H-Ras(V12), but not wild-type H-Ras, were effectively killed after inactivation of NF-κB by SR-IκBα (Fig. 3B). Our results indicate that p53 null cells can be effectively transformed by Ras because this oncogene activates NF-κB to suppress apoptosis.

To examine whether both p53-dependent and -independent mechanisms were operative in the Ras-induced apoptosis, we used the mutant $p53^{135}$ protein, which functions as a dominant negative inhibitor of endogenously expressed wild-type p53 protein (*23*). Because p53 is wild-type in NIH 3T3 cells (*24*), it is possible that p53 contributed to cell death after the expression of oncogenic Ras and SR-IκBα (Fig.1). Parental NIH 3T3 cells were cotransfected with pCMV-LacZ, together with either the vector control (pLTR) or pLTR-$p53^{135}$, and with either the CMV-4 control vector or the SR-IκBα vector. The dominant negative $p53^{135}$ mutant did not inhibit oncogenic Ras–mediated killing after the inactivation of NF-κB (Fig. 3C). The $p53^{135}$ mutant appeared to be functional because it inhibited p53-dependent gene expression in NIH 3T3 cells after doxorubicin treatment (*14*) and because it blocked p53-dependent apoptosis (*25*). These results indicate that NF-κB activity is required to inhibit p53-independent programmed cell death mediated by oncogenic Ras.

Our data indicate that oncogenic Ras elicits both pro- and anti-apoptotic pathways, with the latter pathway being domi-

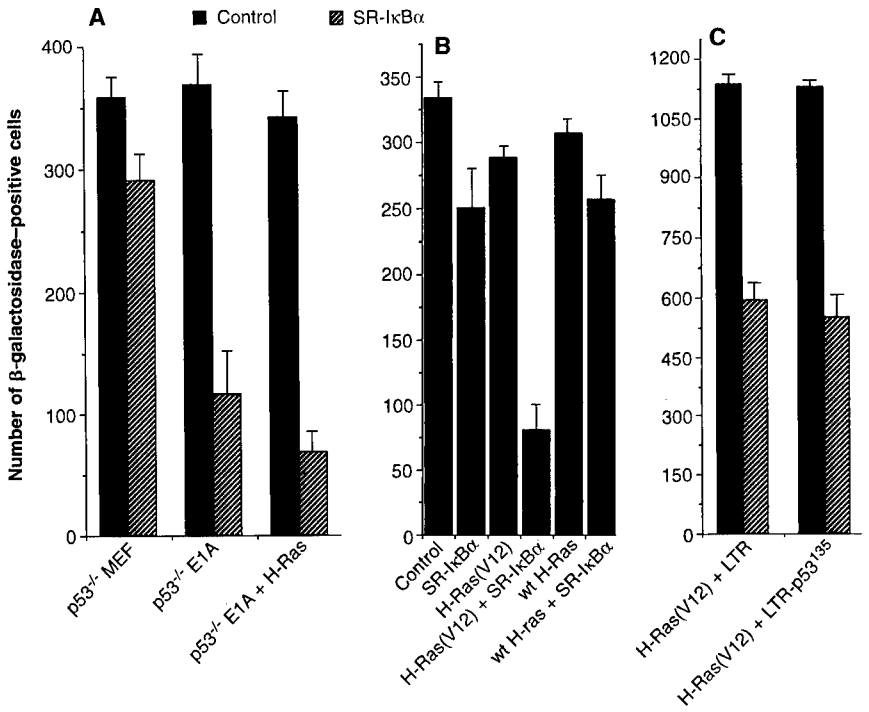


Fig. 3. Ras-mediated apoptosis in cells lacking p53. (**A**) Requirement of NF-κB in E1A and E1A plus H-Ras(V12)–transformed $p53^{-/-}$ fibroblasts for cell survival. $p53^{-/-}$ MEFs (passage 5) and $p53^{-/-}$ MEFs transformed with either E1A or E1A plus activated H-Ras(V12) were transfected with the pCMV-LacZ (0.2 μg) and with the empty vector control, or with the SR-IκBα (1 μg each per 24-well plate). After 48 hours cells were fixed and then stained, and β-Gal–expressing cells were counted. The data are representative of three independent experiments performed in triplicate. (**B**) Ras-mediated p53-independent cell killing. $p53^{-/-}$ MEFs were transfected with the pCMV-LacZ together with either the empty expression vector control or with the various expression constructs indicated. Cells were stained for β-Gal activity 48 hours after transfection. Data are shown as the mean ± SD and are representative of two experiments performed in triplicate. (**C**) Failure of dominant negative p53 to prevent oncogenic Ras–mediated killing after inactivation of NF-κB. Parental NIH 3T3 cells were transfected with the pCMV-LacZ together with either the vector control (pLTR) or with pLTR-$p53^{135}$, which encodes the dominant negative p53 mutant. Additionally, cells were transfected with either the empty expression vector control (pCMV-4) or with SR-IκBα. β-Gal expression assays were performed. Data are shown as the mean ± SD of two independent experiments performed in triplicate. Bars are shaded as in (A).

nant in immortalized rodent cells. Consistent with this idea, Ras has been shown to induce programmed cell death (*26*) and to suppress apoptosis induced by Myc and E1A (*27*, *28*). Our studies demonstrate that the ability of activated Ras to transform p53 null cells is dependent on the ability of this oncogene to activate NF-κB. Thus, there are cell death pathways (independent of p53) that can be initiated by the Ras oncogene after the inactivation of NF-κB. Additional studies indicate that the cell killing induced by Ras is independent of Raf-1 and its signaling cascade (*14*). Consistent with this notion, we found that 3T3 R-Ras(V38) cells were killed equally as well as 3T3 H-Ras(V12) cells after expression of the SR-IκBα construct (Fig. 1). Because R-Ras does not interact with the serine-threonine Raf-1 kinase (*29*), this is consistent with the hypothesis that Raf is not involved in Ras-mediated killing after NF-κB inactivation. Our data demonstrate that oncogenic Ras elicits an apoptotic pathway that is suppressed by the parallel activation of NF-κB. Because a complex array of anti-apoptotic proteins are expressed in tumors, the activation of NF-κB by Ras mutation may play a critical role at the earliest level of tumorigenesis.

REFERENCES AND NOTES

1. J. L. Bos, *Mutat. Res.* **195**, 255 (1988); F. McCormick, *Curr. Opin. Biotechnol.* **7**, 449 (1996); G. J. Clark and C. J. Der, in *GTPases in Biology I*, B. F. Dickey and L. Birnbaumer, Eds. (Springer-Verlag, Berlin, 1993), pp. 259–288; I. G. Macara, K. M. Lounsbury, S. A. Richards, C. McKiernan, D. Bar-Sagi, *FASEB J.* **10**, 625 (1996).
2. R. Khosravi-Far *et al.*, *Mol. Cell. Biol.* **16**, 3923 (1996); T. Joneson, M. A. White, M. H. Wigler, D. Bar-Sagi, *Science* **271**, 810 (1996); R.-G. Quin, F. McCormick, M. Symons, *Nature* **374**, 457 (1995); G. C. Prendergast *et al.*, *Oncogene* **10**, 2289 (1995); R.-G. Qiu, J. Chen, F. McCormick, M. Symons, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11781 (1995); G. J. Clark, J. K. Westwick, C. J. Der, *J. Biol. Chem.* **272**, 1677 (1997).
3. B. Binetruy, T. Smeal, M. Karin, *Nature* **351**, 122 (1991); T. Smeal *et al.*, *ibid.* **354**, 494 (1991); B.-S. Yang *et al.*, *Mol. Cell. Biol.* **16**, 538 (1996); M. Colman and M. Ostrowski, *Nucleic Acids Res.* **24**, 1971 (1996); M. Karin, *J. Biol. Chem.* **270**, 16483 (1995); O. Coso *et al.*, Cell **81**, 1137 (1995); A. Minden, A. Lin, F. Claret, A. Abo, M. Karin, *ibid.*, p. 1147.
4. S. Langer, D. Bortner, M. Roussel, C. Sherr, M. Ostrowski, *Mol. Cell. Biol.* **12**, 5355 (1992); M. Granger-Schnarr, E. Benusiglio, M. Schnarr, P. Sassone-Corsi, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4236 (1992); A. Lloyd, N. Yancheva, B. Wasylyk, *Nature* **352**, 635 (1991); M. D. Sklar *et al.*, *Mol. Cell. Biol.* **11**, 3699 (1991).
5. R. Johnson, B. Spiegelman, D. Hanahan, R. Wisdom, *Mol. Cell. Biol.* **16**, 4504 (1996).
6. T. Finco and A. Baldwin, *J. Biol. Chem.* **268**, 17676 (1993).
7. T. S. Finco *et al.*, *ibid.* **272**, 24113 (1997).
8. C.-Y. Wang, M. Mayo, A. Baldwin, *Science* **274**, 784 (1996).
9. A. Beg and D. Baltimore, *ibid.*, p. 782; D. Van Antwerp, S. J. Martin, T. Kafri, D. Green, I. Verma, *ibid.*, p. 787; M. Arsura, M. Wu, G. Sonenshein, *Immunity* **5**, 31 (1996); Z.-G. Liu, H. Hsu, D. Goeddel, M. Karin, *Cell* **87**, 565 (1996).
10. A. Baldwin, *Annu. Rev. Immunol.* **14**, 649 (1996).
11. G. Williams, *Cell* **65**, 1097 (1991); C. Thompson, *Science* **267**, 1456 (1995).
12. J. Brockman *et al.*, *Mol. Cell. Biol.* **15**, 2809 (1995); K. Brown *et al.*, *Science* **267**, 1485 (1995); E. Traenckner *et al.*, *EMBO J.* **14**, 2876 (1995); J. DiDonato *et al.*, *Mol. Cell. Biol.* **16**, 1295 (1996).
13. V. Palombello, O. Rando, A. Goldberg, T. Maniatis, *Cell* **78**, 773 (1994); Z. Chen *et al.*, *Genes Dev.* **9**, 1586 (1995).
14. M. W. Mayo and A. S. Baldwin Jr., unpublished data.
15. S. McCarthy, M. Samuels, C. Pritchard, J. Abraham, M. McMahon, *Genes Dev.* **9**, 1953 (1995).
16. W. Bursch, L. Kleine, M. Tenniswood, *Biochem. Cell Biol.* **68**, 1071 (1990); J. Kerr, C. Winterford, B. Harmon. *Cancer* **73**, 2013 (1994).
17. M. Serrano, A. Lin, M. McCurrach, D. Beach, S. Lowe, *Cell* **88**, 593 (1997).
18. N. Tanaka *et al.*, *ibid.* **77**, 829 (1994).
19. M. Serrano *et al.*, *ibid.* **85**, 27 (1996).
20. M. L. Schmitz *et al.*, *Mol. Cell. Biol.* **16**, 4052 (1996).
21. S. Lowe, H. Ruley, T. Jacks, D. Housman, *Cell* **74**, 957 (1993).
22. M. Debbas and E. White. *Genes Dev.* **7**, 546 (1993).
23. D. Michalovitz, O. Halevy, M. Oren, *Cell* **62**, 671(1990).
24. M. L. Quo, Y. W. Chou, Y. P. Chau, T. S. Huang, *Mol. Carcinog.* **18**, 221 (1997).
25. M. Holmes-McNary and A. S. Baldwin Jr., unpublished data.
26. C. Chen and D. Faller, *Oncogene* **11**, 1487 (1995); H.-J. Lin, V. Eviner, G. Prendergast, E. White, *Mol. Cell. Biol.* **15**, 4536 (1997); H.-G. Wang *et al.*, *J. Cell. Biol.* **129**, 1103 (1995).
27. A. Kauffmann-Zeh *et al.*, *Nature* **385**, 544 (1997).
28. H.-J. Lin, V. Eviner, G. Prendergast, E. White, *Mol. Cell. Biol.* **15**, 4536 (1995).
29. C. Herrmann, G. Horn, M. Spaargaren, A. Wittinghofer, *J. Biol. Chem.* **271**, 6794 (1996); S. Huff, L. Quilliam, A. Cox, C. Der, *Oncogene* **14**, 133 (1997); B. Marte, P. Rodriguez-Viciana, S. Wennstrom, P. Warne, J. Downward, *Curr. Biol.* **7**, 63 (1997).

30. We thank Y. Kaziro for permission to use the Rat-1: iRas cells and M. McMahon for providing the cell line; D. Ballard for the super-repressor IκBα construct; M. Ostrowski for the Ets-transformed cell line; S. Drouin for immunoblotting; L. Arnold and J. Watson for FACS analysis; E. Reap for helpful discussions; and S. Earp and D. Guttridge for reading the manuscript and for helpful suggestions. Supported by NIH grant CA72771 and by the Department of the Army grant DAMD17-94-J-4053 to A.S.B. C.J.D. and S.W.L. are supported by NIH grants CA52072 and CA13106, respectively. M.W.M. is supported by NIH postdoctoral fellowship 1F32-CA69790. C.-Y.W. is supported by a Dentist Scientist Award from NIH.

20 March 1997; accepted 30 October 1997

Reprint Series
1 November 1996, Volume 274, pp. 784–787

SCIENCE

TNF- and Cancer Therapy-Induced Apoptosis: Potentiation by Inhibition of NF-κB

Cun-Yu Wang, Marty W. Mayo, and Albert S. Baldwin Jr.*

TNF- and Cancer Therapy–Induced Apoptosis: Potentiation by Inhibition of NF-κB

Cun-Yu Wang, Marty W. Mayo, Albert S. Baldwin Jr.*

Many cells are resistant to stimuli that can induce apoptosis, but the mechanisms involved are not fully understood. The activation of the transcription factor nuclear factor–kappa B (NF-κB) by tumor necrosis factor (TNF), ionizing radiation, or daunorubicin (a cancer chemotherapeutic compound), was found to protect from cell killing. Inhibition of NF-κB nuclear translocation enhanced apoptotic killing by these reagents but not by apoptotic stimuli that do not activate NF-κB. These results provide a mechanism of cellular resistance to killing by some apoptotic reagents, offer insight into a new role for NF-κB, and have potential for improvement of the efficacy of cancer therapies.

Observations that NF-κB (*1*) is activated by certain apoptotic stimuli has led to the speculation that this transcription factor may mediate aspects of programmed cell death. An anti-apoptotic function of NF-κB is also suggested, however, because mice that lack the NF-κB p65/RelA gene die embryonically from extensive apoptosis within the liver (*2*). Many cells that respond to TNF, a strong activator of NF-κB, are also resistant to cell killing, which is enhanced in the presence of protein synthesis inhibitors (*3*). We investigated, therefore, whether the transcription factor NF-κB is protective against apoptotic killing

induced by TNF in a model cell system. We initiated our studies using the human fibrosarcoma cell line HT1080, which is relatively resistant to killing by TNF (*4*). To potentially block the activation of NF-κB in response to TNF stimulation, we established an HT1080 cell line (HT1080I) expressing a super-repressor form of the NF-κB inhibitor IκBα. The super-repressor IκBα contains serine-to-alanine mutations at residues 32 and 36, which inhibit signal-induced phosphorylation (*5*) and subsequent proteasome-mediated degradation of IκBα (*6*). This mutant IκBα protein acts as a super-repressor because it binds to NF-κB and inhibits DNA binding as well as nuclear translocation but is unable to respond to cellular signals such as those induced by TNF (*5*, *6*). A control line (HT1080V) was established that contained the empty vector and the hygromycin selectable marker. TNF-α–induced NF-κB activation, as measured by DNA binding of nuclear extracts, was effectively blocked by the super-repressor IκBα in HT1080I cells as compared with activation in the control cell line [(*4*) and below].

TNF-α is more effective at inducing apoptosis in the IκBα super-repressor–expressing cells (HT1080I) than in the control cell line (HT1080V) (Fig. 1A). Similar results were obtained with pooled clones of HT1080V or HT1080I cells (*4*), which indicates that the results we obtained were because of the overexpression of the super-repressor IκBα and were not due to clonal variation. That cells were killed by apoptosis was confirmed by the use of the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, which measures DNA strand breaks and is diagnostic for cells undergoing apoptosis. Apoptosis was observed only in the HT1080I cells treated with TNF (Fig. 1B). Other cells were more sensitive to TNF-α killing when the IκBα super-repressor was expressed, which shows that the results were not unique to the HT1080 cells (*4*). Thus, expression of a super-repressor form of IκBα potently enhanced the ability of TNF to initiate apoptosis in a variety of cells that are normally resistant to this cytokine, which suggests that the activation of NF-κB by TNF is protective.

To exclude the possibility that the expression of the super-repressor form of IκBα leads to a function that is different from the inhibition of NF-κB, we confirmed the requirement for NF-κB in inhibition of TNF-induced apoptosis. The pretreatment of HT1080V cells with interleukin-1 (IL-1, an activator of NF-κB that does not initiate apoptosis) blocked the subsequent killing of these cells induced by combined cycloheximide (CHX) and TNF treatment (Fig. 2A). As a control and to determine that it was NF-κB that was responsible for the protection, we found that IL-1 had no protective effect on the HT1080I cell line, in which NF-κB activation is blocked (*4*) by the expression of the super-repressor IκBα (Fig. 2A). IL-1 is known to block TNF-mediated killing (*3*). We determined whether a proteasome inhibitor would enhance cell killing of HT1080 cells in response to TNF treatment, because the degradation of IκBα is controlled by the proteasome after inducible phosphorylation and subsequent ubiquitination (*6*). Proteasome inhibitors of the peptide aldehyde category are potent inhibitors of NF-κB activation (*6*). In a dose-dependent fashion, the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H) strongly enhanced the killing of HT1080V cells in response to TNF (*4*).

If NF-κB inhibition was critical for making cells vulnerable to TNF killing, then overexpression of NF-κB subunits should restore protection against cell killing in the HT1080I model. We transfected vectors encoding the p50 and RelA/p65 subunits of NF-κB or the empty cytomegalovirus (CMV) vector control into the HT1080I cells and stimulated them with TNF. As expected, the vector alone did not provide protection against cell killing induced by TNF (Fig. 2B). However, expression of the NF-κB p50 and RelA/p65 subunits provided protection against TNF-induced apoptosis, indicating that it is NF-κB that is blocked by the super-repressor IκBα and that NF-κB expression blocks programmed cell death. Additional evidence that NF-κB is required for protection against cell killing induced by TNF is shown by the fact that embryonic fibroblasts from RelA/p65 null mice (*2*) are killed by TNF with a much higher frequency than are those from wild-type animals (*4*, *7*).

Many cancer therapies function to kill transformed cells through apoptotic mechanisms; resistance to apoptosis provides protection against cell killing initiated by these therapies (*8*). To determine if other apoptotic stimuli activate NF-κB and whether NF-κB is protective against these stimuli, we analyzed ionizing radiation–, daunorubicin-, and staurosporine-treated cells. Ionizing radiation is known to activate NF-κB in several cell types (*9*). We therefore investigated whether

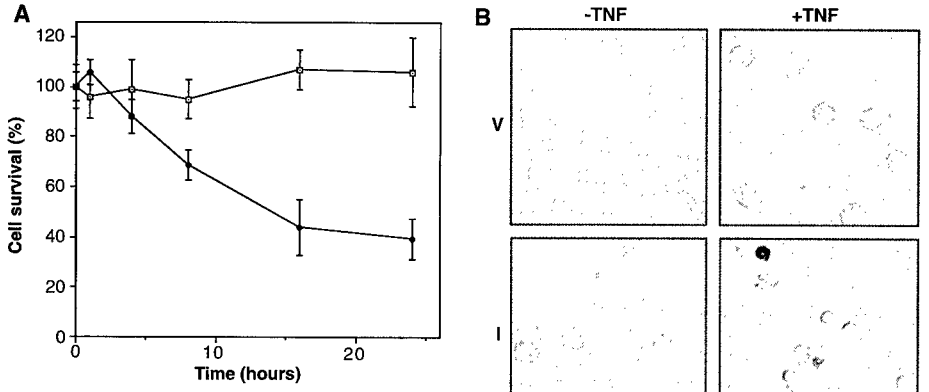


Fig. 1. Expression of the super-repressor IκBα overcomes the block to TNF-mediated apoptosis. (**A**) HT1080 fibrosarcoma cells were cotransfected with the pCMV empty vector or with same vector containing a cDNA encoding the super-repressor IκBα and with the pCEP4 vector for hygromycin B selection (400 μg/ml). Transfection was by the lipofectamine protocol (Gibco/BRL). HT1080I expresses the super-repressor IκBα, and HT1080V contains the empty expression vector. IκBα levels were determined by immunoblotting (ECL, Amersham) of equivalent amounts of protein from the different cells with an antibody to human IκBα (Rockland, Boyertown, Pennsylvania). Expression of the super-repressor IκBα in HT1080 cells efficiently blocked TNF-stimulated NF-κB nuclear translocation, as determined by electrophoretic mobility-shift assay (EMSA) (*4*). Either HT1080V cells (open squares) or HT1080I cells (solid diamonds) were treated with TNF-α (20 ng/ml) for varying times, and surviving cells were quantified by crystal violet assay (*19*). Data shown are the mean of three independent experiments ± SD, and the percentage cell survival was defined as the relative number of TNF-treated versus untreated cells. (**B**) Detection of TNF-induced apoptosis by TUNEL staining (*20*). HT1080V (V) or HT1080I (I) cells were either untreated (−TNF) or were stimulated (+TNF) with TNF-α (50 ng/ml) for 7 hours and then fixed with 4% paraformaldehyde. The staining was done according to the manufacturer's instructions (Boehringer Mannheim). Positive cells show the condensed morphology typical of apoptotic cells.

C.-Y. Wang, Curriculum in Genetics and Molecular Biology and Department of Endodontics, School of Dentistry, University of North Carolina, Chapel Hill, NC 27599–7295, USA.
M. W. Mayo, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599–7295, USA.
A. S. Baldwin Jr., Lineberger Comprehensive Cancer Center, Curriculum in Genetics and Molecular Biology, and Department of Biology, University of North Carolina, Chapel Hill, NC 27599–7295.
*To whom correspondence should be addressed at Lineberger Comprehensive Cancer Center, Campus Box 7295, University of North Carolina, Chapel Hill, NC 27599, USA.

ionizing radiation, the chemotherapeutic compound daunorubicin, and staurosporine activated NF-κB in the HT1080V cells and in the HT1080I cells. Both daunorubicin and ionizing radiation activated NF-κB (Fig. 3A). HT1080I cells were blocked by these two stimuli in their ability to activate NF-κB (Fig. 3A), which is consistent with the expression of the super-repressor IκBα. In contrast, staurosporine was not effective at NF-κB activation (Fig. 3A). We then tested whether ionizing radiation–, daunorubicin-, and staurosporine-induced cell killing can be enhanced by the inhibition of NF-κB activity. For these experiments, we used the HT1080V control fibrosarcoma cells and the HT1080I derivative that expresses the super-repressor form of IκBα. Apoptotic stimuli that induce NF-κB, namely daunorubicin (Fig. 3B) and ionizing radiation (Fig. 3C), are enhanced in their ability to kill the HT1080I cells. However, apoptosis induced by staurosporine is not enhanced by the expression of IκBα (*4*), which is consistent with the observation that staurosporine does not effectively activate NF-κB (Fig. 3A). Thus, the activation of NF-κB is part of the cellular response to a variety of genotoxic agents, and under stress-induced conditions, this transcription factor provides significant protection against apoptosis.

Our data indicate that the activation of NF-κB by TNF, ionizing radiation, and daunorubicin provides protection against apoptotic cell killing induced by these stimuli. Distinct signaling pathways initiated by TNF engagement of its receptor lead to activation of both apoptosis and NF-κB, and NF-κB does not play a positive role in the induction of apoptosis (*10*). In the case of ionizing radiation and daunorubicin, the activation of apoptosis appears to be initiated by ceramide production (*11*) and the cytotoxic effects of TNF have been reported to require ceramide activation (*12*). Ceramide alone has been shown to lead to apoptosis (*13*), but the details of this apoptotic pathway are not fully understood. In each of these three cases, the apoptotic stimulus also leads to an inhibition of apoptosis through the activation of NF-κB. It should be noted that several groups have suggested that NF-κB may function pro-apoptotically under some conditions and in certain cell lines (*14*). The mechanism whereby NF-κB protects cells against apoptosis is presently unclear. Because cell killing by TNF and other apoptotic agents is enhanced by the protein synthesis inhibitor CHX, the activation of NF-κB probably functions to transcriptionally up-regulate a gene or group of genes encoding proteins involved in protection against cell killing.

Growing evidence indicates that a variety of anticancer agents kill through programmed cell death. Resistance to anticancer therapies appears to be mediated by resistance to apoptosis (*8*). Our data show that several anticancer agents may be less effective at inducing programmed cell death because of their concomitant activation of NF-κB. Another cancer therapy, etoposide, activates NF-κB (*15*) and our preliminary data indicate that cell killing by vincristine is augmented by the inhibition of NF-κB (*4*). Therefore, approaches that inhibit nuclear translocation of NF-κB, including gene therapy delivery of the super-repressor IκBα or the use of a variety of agents that block NF-κB function (such as proteasome inhibitors), may prove to be highly beneficial in the treatment of tumors when combined with standard anticancer therapies. In fact, glucocorticoids, which are widely used as immune and inflammatory suppressants and inhibit NF-κB (*16*), are used as part of a therapy for certain hematological malignancies (*17*). It may be, therefore, that the function of glucocorticoids in these therapies is to inhibit NF-κB, potentiating killing by the other chemotherapeutic compounds. Thus, combined therapy that inhibits NF-κB func-

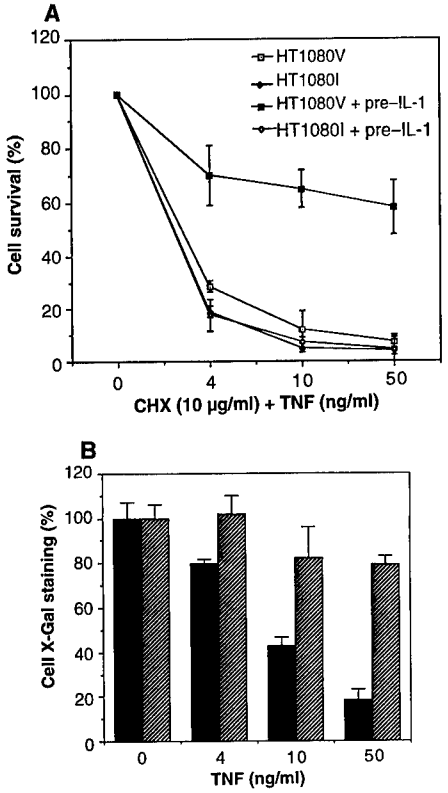


Fig. 2. NF-κB is a suppressor of TNF-induced apoptosis. (**A**) IL-1 pretreatment inhibits TNF + CHX–induced apoptosis in HT1080V cells. HT1080V or HT1080I cells were preincubated with IL-1β (10 ng/ml) (R&D Systems) for 5 hours as indicated (+pre–IL-1) or were left untreated. After the incubation, cells were treated with TNF-α at different concentrations (as indicated) and with CHX (10 μg/ml). Surviving cells were quantified by the crystal violet assay described above. (**B**) Expression of the p50 and RelA/p65 NF-κB subunits restores cell resistance to TNF killing. HT1080I cells (expressing the super-repressor IκBα) were either cotransfected with pcDNA3-lacZ (Invitrogen) and pCMV-p65 (2 μg) and pCMV-p50 (2 μg) (hatched bars) or with lacZ and empty vectors (solid bars) by the lipofectamine protocol. After 40 hours, cells were treated with different concentrations of TNF-α for an additional 24 hours. The results are from the mean ± SD of two experiments. X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

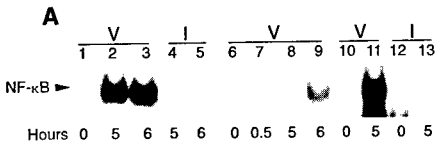
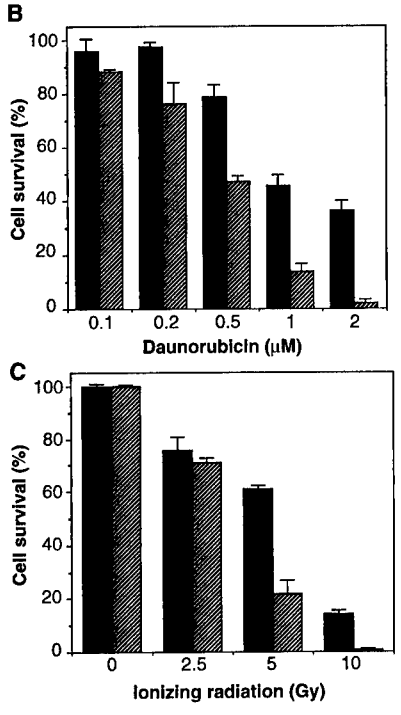


Fig. 3. Activation of NF-κB by ionizing radiation or daunorubicin protects against apoptosis induced by these cancer therapies. (**A**) Daunorubicin and ionizing radiation induce nuclear translocation of NF-κB. Either HT1080V (V) or HT1080I (I) cells were treated with 1 μM daunorubicin (Sigma) or 50 nM staurosporine (Sigma) or were irradiated [at 5 grays (Gy)] for the indicated times. EMSA was performed as previously described (*21*). Lanes 1 through 5, daunorubicin; lanes 6 through 9, staurosporine; and lanes 10 through 13, ionizing radiation. (**B** and **C**) The overexpression of super-repressor IκBα enhanced cell killing by daunorubicin and ionizing radiation. (B) HT1080V cells (solid bars) or HT1080I cells (hatched bars) were treated with the indicated concentration of daunorubicin for 24 hours. Cell survival was assayed as described in Fig. 1. Data are from the mean of four separate experiments. (C) Five hundred HT1080V or HT1080I cells were plated in six-well plates, and 24 hours later the cells were exposed to ionizing radiation at the indicated doses. Cell clones were counted after 14 days. Each experimental group was performed in triplicate. The results shown here represent three independent experiments and are expressed as the mean ± SD.



tion in the presence of apoptotic stimuli may lower the anti-apoptotic threshold of tumors to provide a more effective treatment against resistant forms of cancer. Additionally, the inhibition of NF-κB function in association with TNF treatment may broaden the limited ability of this cytokine to function in an anti-tumor manner.

Note added in proof. Wu *et al.* (*18*) recently demonstrated that NF-κB blocks apoptosis in B cells.

REFERENCES AND NOTES

1. A. Baldwin, *Annu. Rev. Immunol.* **14**, 649 (1996); I. Verma, J. Stevenson, E. Schwarz, D. Van Antwerp, S. Miyamoto, *Genes Dev.* **9**, 2723 (1995); U. Siebenlist, G. Franzoso, K. Brown, *Annu. Rev. Cell Biol.* **10**, 405 (1994); P. Baeuerle and T. Henkel, *Annu. Rev. Immunol.* **12**, 141 (1994); H. C. Liou and D. Baltimore, *Curr. Opin. Cell Biol.* **5**, 477 (1993).
2. A. Beg, W. Sha, R. Bronson, S. Ghosh, D. Baltimore, *Nature* **376**, 167 (1995).
3. H. Holtmann, T. Hahn, D. Wallach, *Immunobiology* **177**, 7 (1988).
4. C.-Y. Wang and A. S. Baldwin Jr., unpublished data.
5. J. Brockman *et al.*, *Mol. Cell. Biol.* **15**, 2809 (1995); K. Brown *et al.*, *Science* **267**, 1485 (1995); E. Traenckner *et al.*, *EMBO J.* **14**, 2876 (1995); J. DiDonato *et al.*, *Mol. Cell. Biol.* **16**, 1295 (1996).
6. V. Palombello, O. Rando, A. Goldberg, T. Maniatis, *Cell* **78**, 773 (1994); Z. Chen *et al.*, *Genes Dev.* **9**, 1586 (1995).
7. A. A. Beg and D. Baltimore, *Science* **274**, 782 (1996).
8. D. E. Fisher, *Cell* **78**, 539 (1994).
9. S. Singh and M. Lavin, *Mol. Cell. Biol.* **10**, 5279 (1990); M. Brach *et al.*, *J. Clin. Invest.* **88**, 691 (1991).
10. L. Tartaglia and D. Goeddel, *Immunol. Today* **13**, 151 (1992); P. Vandenabeele, W. Declercq, R. Beyaert, W. Fiers, *Trends Cell. Biol.* **5**, 392 (1995); H. Hsu, J. Xiong, D. Goeddel, *Cell* **81**, 495 (1995); H. Hsu, H.-B. Shu, M.-P. Pan, D. Goeddel, *ibid.* **84**, 299 (1996); A. Chinnaiyan, K. O'Rourke, M. Tewardi, V. Dixit, *ibid.* **81**, 505 (1995); M. Muzio *et al.*, *ibid.* **85**, 817 (1996); M. Boldin, T. Goncharov, Y. Goltsev, D. Wallach, *ibid.*, p. 803; H. Hsu, J. Huang, H.-B. Shu, V. Baichwal, D. Goeddel, *Immunity* **4**, 387 (1996).
11. R. Bose *et al.*, *Cell* **82**, 405 (1995); J.-P. Jaffrezou *et al.*, *EMBO J.* **15**, 2417 (1996); A. Haimovitz-Freedman *et al.*, *J. Exp. Med.* **180**, 525 (1994); P. Santana *et al.*, *Cell* **86**, 189 (1996).
12. R. Kolesnick and D. Golde, *Cell* **77**, 325 (1994).
13. L. Obeid, C. Linardic, L. Karolak, Y. Hannun, *Science* **259**, 1769 (1993).
14. M. Jung, Y. Zhang, S. Lee, A. Dritschilo, *ibid.* **268**, 1619 (1995); K.-I. Lin *et al.*, *J. Cell Biol.* **131**, 1149 (1995); S. Grimm, M. Bauer, P. Baeuerle, K. Shulze-Osthoff, *ibid.* **134**, 1 (1996).
15. A. Slater, M. Kimland, S. Jiang, S. Orrenius, *Biochem. J.* **312**, 844 (1995); R. Bessho *et al.*, *Biochem. Pharm.* **48**, 1883 (1994).
16. A. Ray and K. Prefontaine, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 752 (1994); N. Mukaida *et al.*, *J. Biol. Chem.* **269**, 13289 (1994); R. Scheinman *et al.*, *Mol. Cell. Biol.* **15**, 943 (1995); E. Caldenhoven *et al.*, *Mol. Endocrinol.* **9**, 401 (1995); R. Scheinman, P. Cogswell, A. Lofquist, A. Baldwin, *Science* **270**, 283 (1995); N. Auphan, J. DiDonato, C. Rosette, A. Helmberg, M. Karin, *ibid.*, p. 286.
17. V. Devita, S. Hubbard, D. Longo, *Cancer Res.* **47**, 5810 (1987); R. Schartzman and J. Cidlowski, *Endocrinol. Rev.* **14**, 133 (1993).
18. M. Wu *et al.*, *EMBO J.* **15**, 4662 (1996).
19. P. Mehlen, C. Kretz-Remy, X. Preville, A.-P. Arrigo, *EMBO J.* **15**, 2695 (1996).
20. T. Graeber *et al.*, *Nature* **379**, 88 (1996).
21. T. Finco, A. Beg, A. Baldwin, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11884 (1994).

22. We thank I. Verma, A. Beg, and D. Baltimore for discussions and data sharing; D. Ballard for the IκBα super-repressor construct; A. Beg for the RelA/p65 null fibroblasts; W. E. Miller for immunobloting and discussions; S. Earp for suggestions; and M. Trope for support and encouragement. Supported by NIH grant AI35098 and by Department of the Army grant DAMD17-94-J-4053 to A.S.B. C.-Y.W. was supported by a Dentist Scientist Award from NIH, and M.W.M. was supported by NIH postdoctoral fellowship 1F32-CA69790-01.

5 August 1996; accepted 27 September 1996

Selective Activation of NF-κB Subunits in Human Breast Cancer: Potential Role for NF-κB2/p52 and for Bcl-3

Patricia C. Cogswell, Denis C. Guttridge, William K. Funkhouser[#], and Albert S. Baldwin, Jr.[*]
Lineberger Comprehensive Cancer Center
[#]Department of Pathology and Laboratory Medicine
University of North Carolina School of Medicine
Chapel Hill, NC 27599-7295

* To whom correspondence should be addressed at:

22-000 Lineberger Comprehensive Cancer Center
University of North Carolina School of Medicine
Chapel Hill, NC 27599-7295

Phone: (919) 966-3652
FAX: (919) 966-0444

KEY WORDS: NF-κB, breast cancer, Bcl-3

ABSTRACT

Background: Breast cancer is characterized by altered expression of growth factors, growth factor receptors and signal transduction cascades. The ultimate targets of these pathways are transcription factors which are likely to control processes important for malignant transformation and progression as well as metastasis. Members of the NF-κB/Rel transcription factor family have been shown recently to be required for cellular transformation by oncogenic Ras and by other oncoproteins and to suppress transformation-associated apoptosis. Furthermore, NF-κB has been shown to be activated by several oncoproteins including HER2/Neu, a receptor tyrosine kinase often expressed in human breast cancer. *Methods*: Both human breast cancer cell lines, human breast tumors and normal adjacent tissue were analyzed by gel mobility shift assay, immunoblotting of nuclear extracts and immunohistochemistry for activation of NF-κB. Furthermore, RNA levels for NF-κB activated genes were analyzed in order to determine if NF-κB is functionally active in human breast cancer. *Results*: Our data indicate that the p65/RelA subunit of NF-κB is activated in breast cancer cell lines. However, breast tumors exhibit an absence or low level of nuclear p65/RelA but show activated c-Rel, p50 and p52 as compared to nontumorigenic adjacent tissue. Additionally, the IκB homolog Bcl-3, which functions to stimulate transcription with p50 or p52, was also activated in breast tumor tissues. There was no correlation between estrogen receptor status and levels of nuclear NF-κB complexes. Transcripts of NF-κB-regulated genes were found elevated in breast tumors as compared to adjacent tissue, indicating functional NF-κB activity. *Conclusions*: These data suggest a direct role for a subset of NF-κB and IκB family proteins, particularly NF-κB2/p52 and Bcl-3, in human breast cancer. Additionally, the activation of functional NF-κB in these tumors likely involves a signal transduction pathway distinct from that utilized by cytokines.

INTRODUCTION

As with virtually all malignancies, breast cancer is associated with dysregulated expression of growth factors and growth factor receptors and kinases associated with growth factor signaling pathways (*1,2*). The ultimate downstream effectors of these pathways are transcription factors. Thus, dysregulated transcription is likely to play an important role in cancer since it has been shown that the transcription factors c-jun, ets, c-myc and NF-κB are each required for cellular transformation controlled by oncogenic Ras alleles (*3-6*). The ultimate gene targets of these transcription factors that contribute to oncogenesis are poorly understood.

NF-κB is a transcription factor that is known to play an important role in controlling immune and inflammatory responses (reviewed in (*7,8*)). There are presently five members of the NF-κB/Rel family: NF-κB1/p50, NF-κB2/p52, c-Rel, RelA/p65 and RelB. The p50 and p52 NF-κB subunits are derived from larger precursor products, p105 and p100 respectively, or from differential translation of their mrRNAs. The classic form of NF-κB, the heterodimer of the p50 and p65 subunits, is normally retained in the cytoplasm through interactions with inhibitor proteins IκBα and IκBβ. Inductive stimuli (TNFα, IL-1, bacterial endotoxin, etc.) lead to the phosphorylation and degradation of IκB, allowing NF-κB to enter the nucleus and regulate gene expression (*7,8*). Bcl-3, a member of the IκB family, functions to stimulate transcription through interactions with the p50 or p52 NF-κB subunits (*9,10*) and to increase nuclear levels of p50 homodimers (*11*). Gene knockout studies confirm the important roles of these transcription factors in immune function and indicate that the RelA/p65 subunit is required to suppress liver cell apoptosis controlled by an unknown process (*8*). Additionally, we and others (*12-16*) have shown that NF-κB suppresses apoptosis initiated by tumor necrosis factor, chemotherapy or radiation.

Evidence for a role of NF-κB in oncogenesis is extensive. The founding member of the NF-κB, c-Rel, is the cellular homolog of v-Rel, the transforming gene of avian reticuloendotheliosis virus (*7*). One of the NF-κB family genes, NF-κB2/p52 (lyt-10), is found translocated in some lymphoid neoplasms (*17*) as is the IκB family member Bcl-3 (*18*). Furthermore, several

oncoproteins, including HER2/Neu, Ras and Bcr-Abl, are known to activate NF-κB and NF-κB is required for transformation induced by Ras and Bcr-Abl (*6,19,20*). Importantly, NF-κB activation suppresses a transformation-associated apoptosis associated with oncogenic Ras expression (*21*). Expression of the RelA/p65 subunit is required for cell growth of Hodgkin's lymphoma (*22*). NF-κB expression has been analyzed in human breast cancer cell lines and in breast cancer tissue. Dejardin et al. (*23*) found that the 100 kD precursor of the NF-κB2/p52 subunit was overexpressed in a majority of breast cancer samples and in cancer cell lines, suggesting a role for the precursor in retaining classic forms of NF-κB in the cytoplasm. Nakshatri et al. (*24*) found that NF-κB RelA/p65 is activated (i.e., nuclear) in the majority of human breast cancer cell lines and that the relative level of NF-κB was inversely correlated with estrogen receptor (ER) expression. Most recently Sonenshein and colleagues (*25*) found increased levels of NF-κB in human breast cancer samples and that inhibition of NF-κB in a breast cancer cell line led to apoptosis. We have explored the NF-κB subunit composition in human breast cancer and have found that the p50, p52 and c-Rel subunits are found in the nucleus in virtually all samples of breast cancer. Importantly, the p52 subunit was significantly upregulated in tumor tissue as compared to stromal fibroblasts or adjacent, non-tumor breast epithelium. There was no apparent correlation between ER status and NF-κB activation in the tumor samples. Interestingly, the p65 subunit was found not to be activated to a significant level in breast cancer. Furthermore, the IκB homolog Bcl-3, which has been shown to function with p50 and p52 subunits to stimulate transcription (*9,10*), was also found to exhibit increased nuclear levels in human breast cancer as compared to non-tumor tissue. Consistent with this observation, transcripts of known NF-κB-regulated genes were found to be significantly elevated in human breast cancer tissue. These data indicate that NF-κB is functionally active in human breast cancer and that a subset of NF-κB complexes, which apparently does not include the p50/p65 heterodimer, is activated in human breast cancer. These data indicate non-typical mechanisms are involved in activation of functional NF-κB in breast cancer.

MATERIALS AND METHODS

Cell Culture

All cell lines were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Core Facility at the University of North Carolina, Chapel Hill. MCF-7 cells were maintained in DMEM, T47D cells were grown in RPMI 1640 medium containing 1X ITS (insulin-transferrin-sodium selenite; Boehringer Mannheim), BT474 cells were cultured in EMEM with ITS, MDA231 cells were grown in Iscoves medium, and SKBR-3 cells were grown in Macoys5A medium. Growth media contained antibiotics and 10% fetal calf serum obtained from Life Technologies.

Tissue Samples

Lumpectomy and mastectomy samples from breast cancer patients were separated into tumor tissue and normal adjacent tissue by a pathologist. Samples were either quickly snap frozen in liquid nitrogen, or directly embedded in paraffin. A total of 17 samples were obtained, of which 7 were matched sets of both tumor and normal breast tissue as determined by histological analysis.

Cell Extracts and Western Blotting

Nuclear extracts from each cell line were prepared as previously described (*6*). Nuclear extracts from snap frozen tissue sections were prepared by resuspending crushed, frozen samples in sucrose buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes pH 7.5, 2mM EDTA, 0.5 mM EGTA, 14 mM β–mercaptoethanol and 0.1% NP40) followed by dounce homogenization. Nuclei were isolated following double sucrose pelleting through 0.88 M and 1 M sucrose solutions. For western blotting analysis, equal amounts of protein were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were blocked in 5% milk in 1X TBST (Tris Buffered Saline, 0.5% Tween 20) and probed with either anti-p50, anti-Bcl3, anti-β-Actin (Santa Cruz Biotechnology), anti-p65 (Rockland), or anti-p52 (Rockland) each at 1:1000 dilution in

1X TBST. Blots were probed with a secondary antibody conjugated to horseradish peroxidase (Promega Corp.) at 1:10,000 dilution in 1X TBST. Protein bands were visualized with an enhanced chemiluminescence detection system (Amersham Life Science).

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed as described previously (*20*). An oligonucleotide corresponding to an NF-κB site in the *H-2K*^{b} gene (5'-CAGGGCTGGGGATTCCCATCTCCCACAGTTTCACTTC-3') was radiolabeled using α-[^{32}P]dCTP and Klenow fragment of DNA polymerase I (Boehringer Mannheim). For antibody supershift analysis, nuclear extracts were preincubated 15 min at room temperature with 1 μg antiserum before the addition of the radiolabeled gel shift probe. Antibodies used in supershift analysis are identical to those utilized for western blotting described above.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut to 4 μm, and sections were deparaffinized in xylene, rehydrated, and probed using specific Rel antibodies (listed above for western blotting). Staining was performed using the Vectastain ABC immunoperoxidase staining kit in conjugation with 3',3'-diaminobenzidine (Vector Labs). Tissues were counterstained with Mayer's hematoxylin (Sigma), overlaid with permount mounting solution (Fisher Scientific) and placed under coverslip.

RT-PCR

Snap frozen tissue sections were homogenized in 4 M guanidinium isothiocyanate using a Brinkman Polytron (Westbury). RNA was then isolated by centfrifugation through 5.7 M cesium chloride. To produce cDNA, total RNA (4 μg) and random hexamers (200 ng; Life Technologies) were denatured at 80°C for 10 min and incubated for 60 min at 37°C in first strand synthesis buffer containing 10 mM DTT (Life Technologies), with 625 μM dNTP (Boehringer Mannheim), and 400

units of MMLV reverse transcriptase (Life Technologies). Resulting cDNAs were diluted 1:5 in dH_2O and an aliquot was combined with a mixture containing 200 nM of specific oligonucleotides, 250 μm dNTP, 1.5 mM magnesium chloride, 1X PCR buffer, and 2.5 units of Taq polymerase (Life Technologies). Cycle conditions were the following: denaturation, 94°C for 30 sec; annealing, 58°C for 30 sec; and extension, 72°C for 1 min. The amount of input cDNA was adjusted first to obtain equal amounts of β-Actin product. Amplified products ranged from 450-550 bp in length. Forward (1) and reverse (2) primers used for RT-PCR were the following: p65-1, 5'-CGTGGAGGTGTATGATGATGACGTA-3'; P65-2, 5'-CTAGAATCTAGCTGGAGGGAGGCCA-3'; p50-1, 5'-TGGATCCTTCTTTGACTCATACAAT-3'; p50-2, 5'-GTCTGCTGCAGAGCTGCTTGGCGGA-3'; IκBα-1, 5'-GGGACCCTCAGCAGAGAGGAGGACC-3'; IκBα-2, 5'-GCTTTCAGTTGTTGTGATGCTGAGA-3'. All products were amplified for 25 cycles with the exception of p65 which required a total of 35 cycles for visualization. Amplified products were fractionated on 1.5% agarose gels in 1X TBE (Tris Borate-EDTA), visualized by ethidium bromide, transferred to Nytran Plus (Schleicher & Schuell) and hybridized with specific Rel probes, radiolabelled with [α-^{32}P]dCTP (Pharmacia Biotech). Hybridizations were performed for 1 hr at 68°C in QuickHyb Hybridization Solution (Stratagene) as recommended by the manufacturer. Blots were washed once in 2X SSC for 15 min at room temperature followed by two washes in 0.1X SSC for 30 min at 65°C. Exposure times ranged from 1to 2 days.

RESULTS

NF-κB DNA binding is increased in breast cancer cell lines. In many instances, the utilization of immortalized cell lines is widely recognized as an appropriate model to test the function of a particular transcription factor. In order to determine whether NF-κB plays a role in breast cancer, we examined several breast cancer cell lines for the activation of NF-κB. Activation of NF-κB is characterized by an increase in nuclear NF-κB, an event that can be monitored through electrophoretic mobility shift assay (EMSA). We analyzed 5 well characterized breast cancer cell lines: MCF7, T47D, MDA231, SKBR3, and BT474. Nuclear extracts from each of these cell lines were incubated with a radiolabelled oligonucleotide probe containing an NF-κB DNA binding site. Nuclear extracts from SKBR3 cells contain the strongest NF-κB binding complex followed by BT474, MDA231 and MCF7 (Fig. 1A). Nuclear extracts from T47D cells contain very little NF-κB DNA binding activity. Similar data was obtained using different nuclear extract preparations (data not shown). To identify which NF-κB components contribute to this binding activity, we performed supershift analysis with NF-κB specific antibodies. An antibody specific for the p65 subunit of NF-κB, which can recognize p65 homodimers and p50/p65 heterodimers of NF-κB, supershifted complex I and was unable to shift complex II (Fig. 1B). However, both complexes I and II are supershifted when a p50 antibody, which recognizes both p50 homodimers and p50/p65 heterodimers, and a p52 antibody are used. When an antibody specific for c-Rel is added to the binding reaction there appears to be a decrease in DNA binding but no visible supershift complex is formed. The lack of c-Rel binding is not due to the oligonucleotide probe favoring binding of p65 over that of c-Rel, since nuclear extracts prepared from WEHI-231 cells, known to contain high levels of c-Rel binding activity, show that the probe is readily bound by c-Rel (data not shown). These data suggest that there is an increase in NF-κB DNA binding complexes in nuclear extracts of breast cancer cell lines. The greatest NF-κB binding activity occurs with complexes containing the p65 subunit of NF-κB, a classic NF-κB component.

A recent study demonstrated that estrogen receptor (ER) negative breast cancer cell lines contain an increase in constitutive NF-κB DNA binding when compared to ER-positive cell lines

(*24*). In our study we find that the ER negative SKBR3 cell line does show the greatest NF-κB binding complexes (Fig. 1), however NF-κB binding complexes formed in the ER-positive cell line BT474 are only slightly lower. In addition, by western analysis, the ER-positive cell line MCF-7 appears to have the highest expression of both p65 and c-Rel proteins (data not shown). We have also observed by western analysis that the ER-positive T47D cell line, which has very little NF-κB DNA binding activity, appears to have altered or deleted forms of the various NF-κB family member proteins (data not shown). The altered protein expression in these cells is likely contributing to the lack of NF-κB DNA binding observed in EMSA (Fig. 1B), and probably has nothing to do with the ER status in that cell line. The fact that we do not observe a strong correlation with NF-κB and ER activity in these cell lines may be due to the fact that cell lines carried in culture can show great differences in the amount of NF-κB present over time. In addition, slight changes in culture media and the source of the original cell may also contribute to these differences.

Primary tumor cells from patients with breast cancer contain increased NF-κB binding activity. Although we have analyzed NF-κB DNA binding in breast cancer cell lines, we were interested in determining the status of NF-κB activity in cells from primary breast tumors. Seventeen cases of carcinomas of the breast were received from Lineberger Comprehensive Cancer Center Tissue Procurement files. Sixteen were primary breast carcinomas, and one was a local recurrence. Twelve cases were pure ductal, ranging in size from 0.4 - 9 cm in diameter; ranging from grade 1-3. Of the cases for which steroid receptor data are available, 3/8 were ER positive and 3/6 were PR positive. Two cases were mixed ductal and lobular, ranging in size from 0.4 - 5 cm; ranging from grade 1-2. Of the cases for which steroid receptor data are available, 2/2 were ER positive, and 2/2 were PR positive. Two cases were pure lobular, ranging in size from 1.7 cm to diffuse (>20 cm), ranging from nuclear grade 1-2. Of the cases for which steroid receptor data are available, 1/2 were ER positive, and 1/2 were PR positive.

Nuclear extracts from snap frozen tissue sections of primary breast tumors and normal adjacent breast tissue were obtained from nuclei isolated by sucrose pad purification. EMSAs were performed on nuclear extracts and DNA binding activity between tumor (T) and adjacent tissue (A) was analyzed. The results show that all tumor samples contain NF-κB specific DNA binding activity (Fig. 2A). Although patient 1 appears to have the highest level of NF-κB binding all 7 patients show a dramatic increase in NF-κB DNA binding complexes when compared to their nontumorigenic adjacent tissue. Although the absolute levels of NF-κB binding vary between tumorigenic patient samples, all have correspondingly higher binding than their adjacent tissue (Fig. 2A). The level of NF-κB binding does not appear to be related to the estrogen receptor status since both ER+ and ER- samples have high levels of NF-κB binding (Fig. 2A).

The EMSA complexes detected with breast cancer nuclear extracts do not appear to contain the same NF-κB binding complexes as breast cancer cells, since these DNA-protein complexes are faster migrating than the predominant p50/p65 heterodimer complex found in the cell lines (not shown). To identify which components of NF-κB contribute to this binding activity, supershift analysis was performed with nuclear extracts from patient 7. (Fig. 2B). Supershift analysis identified p50, p52 and c-Rel as the predominent NF-κB subunits contained in these binding complexes (Fig. 2B). Suprisingly when a p65 specific antibody which recognizes both p65 homodimers and p50/p65 heterodimers was added to the binding reaction only the very faint upper complex seen is supershifted. This complex appears in only 3 of the 17 patient samples analyzed and thus does not appear to be an important factor in the overall upregulation of NF-κB activity in primary breast tumor cells. From these results we conclude that the increase in NF-κB DNA binding present in the nuclear extracts from breast tumor cells appears to represent an increase in p50, p52 and c-Rel but not p65. Similar results were obtained in all tumor and adjacent samples tested (data not shown). In order to confirm the statistical significance of our previous result, EMSAs were performed on additional patient tumor samples. The results from these patients are demonstrate that NF-κB DNA binding activity is elevated in 10/10 breast tumors examined (data not shown). Thus, we have examined tumors from 17 patients and every patient sample exhibited an

increase in NF-κB binding activity. Suprisingly, this NF-κB does not involve p65, the most commonly analyzed NF-κB component. Although a few patients (3/17) showed an increase in p65 binding, the level of binding is significantly less than exhibited by the major complex. These data indicate that NF-κB family members other than p65 are upregulated in breast tumors.

To correlate the increase in p52, p50 and c-Rel seen in EMSA with actual protein levels, western blot analysis was performed using the nuclear extracts examined in Figure 2. Nuclear proteins were electrophoresed on an SDS polyacrylamide gel, transferred to membrane and probed with a panel of NF-κB specific antibodies. The results are shown in Figure 3. An increase in p50, p52, and c-Rel protein levels is observed in each tumor sample when compared to adjacent nontumorigenic tissue. In agreement with our EMSA data, there was little detectable p65 protein present in either the adjacent or tumor samples from these patients by our standard western procedure. An antibody specific for human β-actin was used to indicate equal loading. This data again differs from data obtained with breast tumor cell lines that show a significant increase in p65 protein in western analysis (data not shown).

Both p50 and p52 proteins appear to be normally transcriptionally inactive, except when found in a complex with the IκB homolog Bcl-3 (*9,10*). Therefore, we probed the breast cancer nuclear extracts for Bcl-3. As shown in Figure 3, Bcl-3 was found to be significantly elevated in each of the tumor samples analyzed as compared to the normal adjacent tissue. These results provide a potential mechanism to explain transcriptional activity associated with nuclear levels of p50 and p52 (see below and see Discussion).

Immunohistochemistry confirms an increase in p50, p52, and c-Rel proteins. To confirm that p50, p52 and c-Rel are present at higher levels of breast tumor samples when compared to their normal adjacent tissues, immunohistochemistry was performed using NF-κB specific antibodies on paraffin embedded sections of normal and tumorigenic breast tissue from patients 1 and 2. The results shown in Figure 4 are from patient 1 and indicate a dramatic increase in p52 and c-Rel protein levels in breast tumors when compared to nontumorigenic adjacent tissue. The

increased staining for c-Rel appears to be in the infiltrating ductal regions as well as in the surrounding stromal cells indicating an overall increase in staining in the tumor section. However, the staining for p52 is specifically increased in the cancerous ductal regions of the tumor section, with very little staining observed in the stromal cells suggesting that the increase in p52 protein is seen primarily in the cancer cells. There is also an increase in staining in the tumor samples when compared to adjacent tissue for a p50 specific antibody. Because of the high cytoplasmic staining for each of the samples, it is difficult to determine whether there is a corresponding increase in nuclear staining. However, our supporting EMSA and western data demonstrate that there is a dramatic increase in nuclear levels of these NF-κB subunits. Staining with an antibody for the p65 subunit of NF-κB indicates that there is little difference in p65 protein levels between adjacent and tumor tissue. To determine whether the increased staining seen with the the p65 antibody is nuclear, an antibody specific for the activated form of p65 (p65-NLS, which recognizes p65 released from IκB) was utilized and no antibody specific staining in either the adjacent or tumor sections is detectable (data not shown). This data supports our previous observations p65 is not nuclear in tumorigenic tissue of patients with breast cancer. Therefore, breast cancer tissues contain increased NF-κB involving the NF-κB subunits p50, p52, and c-Rel as well as the IκB-like protein Bcl-3. The upregulation of p52 appeared to be more specific for tumor cells.

Activation of NF-κB regulated genes in tumorigenic tissue samples. Many of the NF-κB and IκB genes are known to be regulated by NF-κB, thus allowing for the assay for potentially increased κB-dependent gene expression in breast cancer. Thus, the p50 and p52 genes, but not the gene encoding p65, are known to regulated by NF-κB (see (*7,26*)). In order to determine whether the increase in p50 and p52 levels observed in breast cancer tumor tissue was due to an increase in mRNA, additional tumor samples were obtained and RNA analysis was performed. Enzymatic amplification by PCR is well suited for RNA analyses in this study because the limited sample amount we were able to obtain was insufficient for more standard methods of analysis. Using reverse transcriptase-polymerase chain reaction (RT-PCR) under limiting conditions, we generated

amplified products which were electrophoresed on agarose gels. Amplified DNA levels were quantified by Southern analysis. As shown in Figure 5, an increase in mRNA from the p50 and p52 genes is observed in tumor tissue when compared to mRNA levels for these genes in adjacent tissue. Additionally, a dramatic increase in mRNA from the IκBα gene, which is known to be regulated by NF-κB (*7*), is present in tumor samples. When oligos specific for p65 were used, an additional 10 PCR cycles were necessary in order to visualize any product by southern analysis. Consistent with our previous findings, there is no increase in p65 mRNA between adjacent and tumor tissue. The normalization using human β-actin shows equal PCR products for each sample. This data suggests that the increase in protein seen in western analysis for p50 and p52 is caused by an increase in mRNA level due to either the stabilization of the mRNA or, more likely, to an increase in trancription of the NF-κB regulated genes likely through the activation of functional NF-κB.

DISCUSSION

The results we obtained through EMSA and western analysis of nuclear extracts isolated from patients with breast cancer along with immunohistochemical studies revealed a distinct pattern of activation of NF-κB subunits as compared to normal, adjacent tissue. Specifically a different pattern of NF-κB expression was found from that seen in commonly used breast cancer cell lines and from that seen following treatment of cells with cytokines or other NF-κB inducers. Thus, breast cancer cell lines exhibit a constitutive level of NF-κB activation that consists primarily of the p50/p65 heterodimer. Consistent with these findings, we have made the observation in our laboratory that long term culture of a variety of cell lines leads to increased levels of nuclear NF-κB consisting of the p65 subunit (data not shown). However, our data indicate that NF-κB binding complexes containing p50, p52, and c-Rel are activated (i.e., nuclear) in human breast cancers without a corresponding activation of the p65 subunit. With respect to NF-κB and its potential

biological role in breast cancer, our results demonstrate that established breast cancer cell lines may not serve as a reliable model for this disease.

Although p52 and c-Rel levels and binding activities are increased in breast tumors as compared to non-tumorigenic adjacent tissue, significant levels of p50 are found in the nuclei of the adjacent tissue. Interestingly, immunohistochemistical approaches show a dramatic quantitative increase of p52 in the ductal regions of the breast cancer and not in the invading stromal fibroblasts (Fig. 4). Previously, it was shown that cellular levels of the 100 kD precursor of p52 were elevated in a majority of breast cancer samples (*23*). However, corresponding nuclear levels of p52 were not analyzed in these primary samples. Although c-Rel is known to harbor transcriptional activation domains, both the p50 and p52 have not been shown to be transcriptionally active unless complexed with the IκB homolog Bcl-3 (*9,10,27*). Importantly, we find that Bcl-3 nuclear levels are increased in breast cancer tissue as compared to the adjacent levels, which is interesting since it was published recently that Bcl-3 can function to increase nuclear levels of p50 (*11*). Thus, an increase in nuclear Bcl-3 may function with increased levels of p50 and p52 to account for the enhancement in NF-κB dependent gene expression observed in human breast cancer (Figure 5). Since p50 and p52 are not thought to be directly regulated through complexes with IκBα or IκBβ, our data suggest that a pathway is active in breast cancer that functions to increase levels of certain forms of NF-κB that is independent of the normal inductive pathway that involves degradation of one of the IκB subunits. However, we cannot rule out that the increase in nuclear levels of c-Rel found in breast cancer is controlled through a pathway that involves release from a form of IκB. Our data also indicate that levels of mRNAs for p50 and p52 are increased in breast cancer, likely through the transcriptional activation of these genes by active NF-κB complexes (*7,26*). This is likely to contribute to the increase in these NF-κB subunits in breast cancer.

Of the NF-κB subunits found activated in human breast cancer, c-Rel and p52 each have been implicated with oncogenesis (*7,17*). The p100/p52 gene is found translocated and rearranged in certain lymphomas such that it is overexpressed without the normal ankyrin repeats. c-Rel can be transforming when mutated and expressed in retroviral form (*28*). Importantly, we find that Bcl-3

nuclear levels are increased in human breast cancer and Bcl-3 has also been found translocated (t14;19) and overexpressed in certain T-cell lymphomas (*18*). What is the role for elevated NF-κB in human breast cancer? Our recent data indicates that NF-κB activation suppresses a transformation-associated apoptosis (*21*). Thus, NF-κB may serve to suppress a similar apoptosis in developing breast cancer. Consistent with this, Sonenshein and colleague (*25*) found that inhibition of NF-κB in a breast cancer cell line led to enhanced cell death. Future experiments will determine if the forms of NF-κB that are activated in human breast tumors provide cell survival in the tumor tissue and can activate genes known to suppress apoptosis. Additionally, our recent data indicates that NF-κB can promote cell-cycle progression (D.G. and A.B., submitted) and thus, the activation of NF-κB may promote the enhanced cell proliferation and overexpression of certain cyclins that are characteristic of breast cancer (*29*).

NOTES

This research was supported by a grant DAMD-17-4053 from the Department of the Army and by NIH grant CA72771 (to A.S.B). D.G. was supported by a training grant to the Lineberger Comprehensive Cancer Center (NCI T32CA09156-24). We gratefully acknowledge J. Reuther for helpful suggestions on the manuscript and on several different procedures. We also acknowledge the Lineberger Tissue Procurement Facility for providing the tumor specimens.

REFERENCES

(*1*) Dickson RB, Lippman ME. Growth factors in breast cancer. Endocrine Rev. 1995;16:559-589.

(*2*) Resnik JL, Reichart DB, Huey K, et al. Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. Cancer R. 1998;58:1159-1164.

(*3*) Johnson R, Spiegelman B, Hanahan D, et al. Cellular transformation and malignancy induced by ras require c-jun. Mol. Cell. Biol. 1996;16:4504-4511.

(*4*) Langer SJ, Bortner DM, Roussel MF, et al. Mitogenic signaling by colony-stimulating factor 1 and ras is suppressed by the ets-2 DNA-binding domain and restored by myc overexpression. Mol. Cell. Biol. 1992;12:5355-5362.

(*5*) Sklar MD, Thompson E, Welsh MJ, et al. Depletion of c-myc with specific antisense sequences reverses the transformed phenotype in ras oncogene-transformed NIH 3T3 cells. Mol. Cell. Biol. 1991;11:3699-3710.

(*6*) Finco TS, Westwick JK, Norris JL, et al. Oncogenic Ha-Ras-induced signaling activates NF-kappaB transcriptional activity, which is required for cellular transformation. J. Biol. Chem. 1997;272:24113-24116.

(*7*) Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol. 1996;14:649-683.

(*8*) Ghosh S, May MJ, Kopp EB. NF-kB and Rel proteins: evolutionarily conserved mediators of immune responses. Ann. Rev. Immunol. 1998;16:225-260.

(*9*) Bours V, Franzoso G, Azarenko V, et al. The oncoprotein Bcl-3 directly transactivates through kappa B motifs via association with DNA-binding p50B homodimers. Cell 1993;72:729-739.

(*10*) Fujita T, Nolan GP, Liou HC, et al. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev. 1993;7:1354-1363.

(*11*) Watanabe N, Iwamura T, Shinoda T, et al. Regulation of NFKB1 proteins by the candidate oncoprotein BCL-3: generation of NF-kappaB homodimers from the cytoplasmic pool of p50-p105 and nuclear translocation. EMBO J. 1997;16:3609-3620.

(*12*) Arsura M, Wu M, Sonenshein GE. TGF beta 1 inhibits NF-kappa B/Rel activity inducing apoptosis of B cells: transcriptional activation of I kappa B alpha. Immunity 1996;5:31-40.

(*13*) Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 1996;274:782-784.

(*14*) Liu ZG, Hsu H, Goeddel DV, et al. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 1996;87:565-576.

(*15*) Van Antwerp DJ, Martin SJ, Kafri T, et al. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 1996;274:787-789.

(*16*) Wang CY, Mayo MW, Baldwin AS, Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 1996;274:784-787.

(*17*) Chang CC, Zhang J, Lombardi L, et al. Rearranged NFKB-2 genes in lymphoid neoplasms code for constitutively active nuclear transactivators. Mol. Cell. Biol. 1995;15:5180-5187.

(*18*) Michaux L, Dierlamm J, Wlodarska I, et al. t(14;19)/BCL3 rearrangements in lymphoproliferative disorders: a review of 23 cases. Cancer Gen. Cyto. 1997;94:36-43.

(*19*) Galang CK, Garcia-Ramirez J, Solski PA, et al. Oncogenic Neu/ErbB-2 increases ets, AP-1, and NF-kappaB-dependent gene expression, and inhibiting ets

activation blocks Neu-mediated cellular transformation. J. Biol. Chem. 1996;271:7992-7998.

(*20*) Reuther JY, Reuther GW, Cortez D, et al. A requirement for NF-kappaB activation in Bcr-Abl-mediated transformation. Genes Dev. 1998;12:968-981.

(*21*) Mayo MW, Wang CY, Cogswell PC, et al. Requirement of NF-kappaB activation to suppress p53-independent apoptosis induced by oncogenic Ras. Science 1997;278:1812-1815.

(*22*) Bargou RC, Emmerich F, Krappmann D, et al. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J. Clin. Invest. 1997;100:2961-2969.

(*23*) Dejardin E, Bonizzi G, Bellahcene A, et al. Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. Oncogene 1995;11:1835-1841.

(*24*) Nakshatri H, Bhat-Nakshatri P, Martin DA, et al. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol. 1997;17:3629-3639.

(*25*) Sovak MA, Bellas RE, Kim DW, et al. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J. Clin. Invest. 1997;100:2952-2960.

(*26*) Liptay S, Schmid RM, Nabel EG, et al. Transcriptional regulation of NF-kappa B2: evidence for kappa B-mediated positive and negative autoregulation. Mol. Cell. Biol. 1994;14:7695-7703.

(*27*) Schmid RM, Liptay S, Betts JC, et al. Structural and functional analysis of NF-kappa B. Determinants of DNA binding specificity and protein interaction. J. Biol. Chem. 1994;269:32162-32167.

(*28*) Gilmore TD. Rel/NF-kB. Sem. Cancer. Biol. 1997;8:61-129.

(*29*) Sherr CJ. Cancer cell cycles. Science 1996;274:1672-1677.

FIGURE LEGENDS

Figure 1. Analysis of NF-κB in human breast cancer cell lines. **A.** EMSAs were performed with nuclear extracts prepared from MDA231, MCF7, BT474, SKBR3, and T47D breast cancer cell lines. Nuclear extracts (5 μg) were incubated with a ^{32}P-labeled oligonucleotide probe containing an NF-κB binding site (in Methods and Materials section), and complexes were resolved on a 5% nondenaturing polyacrylamide gel. NF-κB complexes I and II are indicated with arrows. The estrogen receptor (ER) status of each cell line is indicated below each lane. **B.** Supershift analysis was performed by preincubating nuclear extract prepared from the SKBR3 cell line with various NF-κB/Rel specific antibodies for 15 min prior to the addition of the oligonucleotide probe. Arrows placed on the right side of the panel indicate supershifted complexes.

Figure 2. NF-κB DNA binding activity is elevated in breast tumors. **A.** EMSAs were performed on sucrose pad purified nuclear extracts prepared from frozen tissue obtained in 7 patients with breast cancer . Equivalent amounts of protein (2 μg) were used to identify DNA binding activity in tumor (T) when compared to normal adjacent tissue (A). Nuclear extracts were incubated with a ^{32}P-labeled oligonucleotide probe containing an NF-κB binding site. **B.** Supershift analysis was performed on nuclear extracts isolated from tumor samples of patient 7. Extracts were preincubated for 15 min with Rel specific antibodies prior to the addition of probe. Upper arrows indicate supershifted complexes.

Figure 3. Levels of nuclear NF-kB proteins are elevated in breast tumors. Sucrose pad purified nuclear extracts (25 μg) from patients 4-7 were analyzed by western blot analysis. Proteins were fractionated on SDS polyacrylamide gel, transferred to nitrocellulose and incubated with antibodies specific for p65, p50, p52, c-Rel and Bcl3. Normal adjacent tissue (A) and tumor tissue (T) are indicated for each patient. Blots were normalized to the level of β-actin protein.

Figure 4. Immunohistochemistry analysis of breast tumor tissue for NF-κB. Sections of paraffin-embedded tumor (T) and normal adjacent tissue (A) from patient 1 were probed with p65, p50, p52, or c-Rel specific antibodies, and bound antibodies were visualized using immunoperoxidase detection. Following antibody specific staining, sections were counterstained with hematoxylin.

Figure 5. Increased message levels of p52, p50, IκBα but not p65 are seen in breast tumors. Total RNA (4 μg) was isolated from tumor and normal adjacent tissue of patients 1 and 7. RT-PCRs were performed to generate partial cDNA transcripts encoding the NF-κB family members p65, p50, p52, and IκBα. RT-PCR products were resolved on agarose gels and detected via Southern blotting using independently derived cDNA probes. NF-κB products were amplified 25 cycles with the exception of p65 which required an additional 10 cycles for visualization. Exposure times for southern blots ranged from 24-48h. In all cases, RT-PCR products were of the size predicted by their nucleotide sequences.

Figure 1

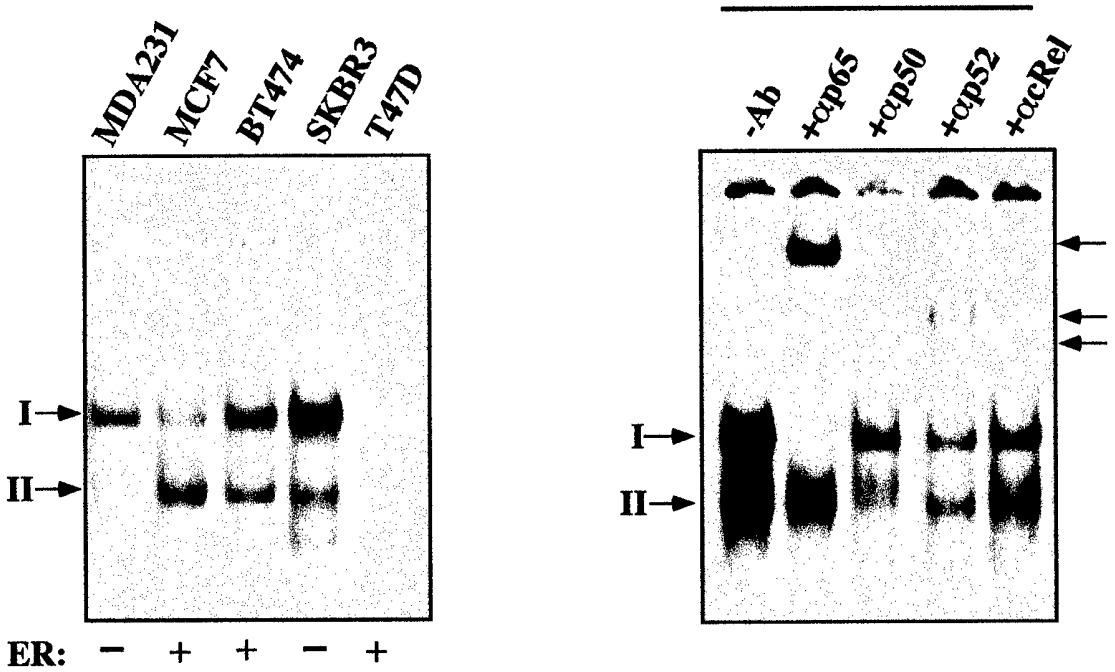


Figure 2A

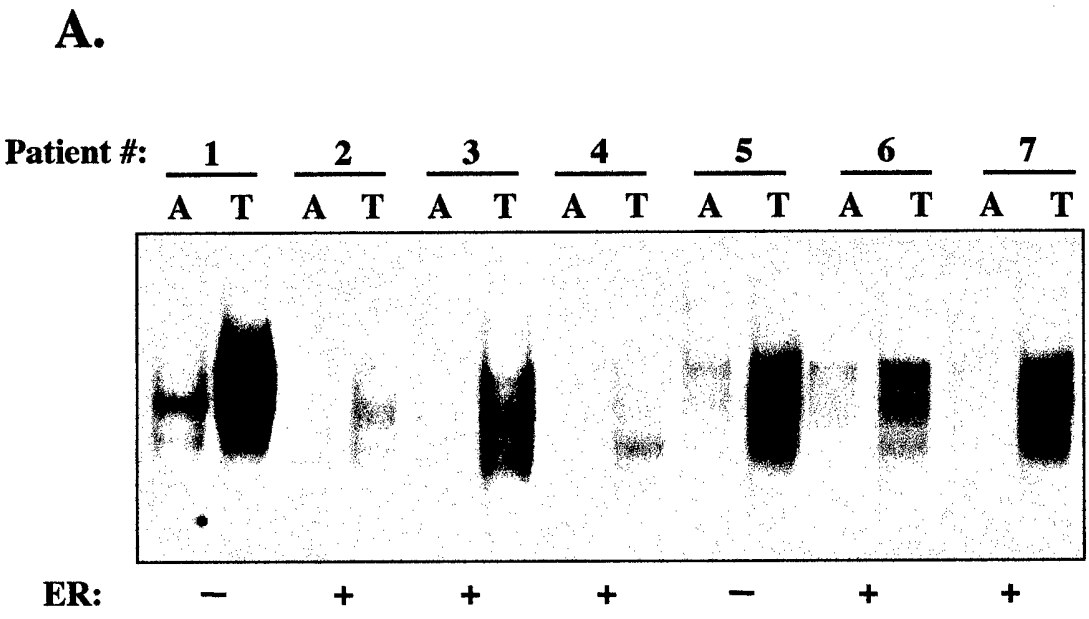


Figure 2B

B.

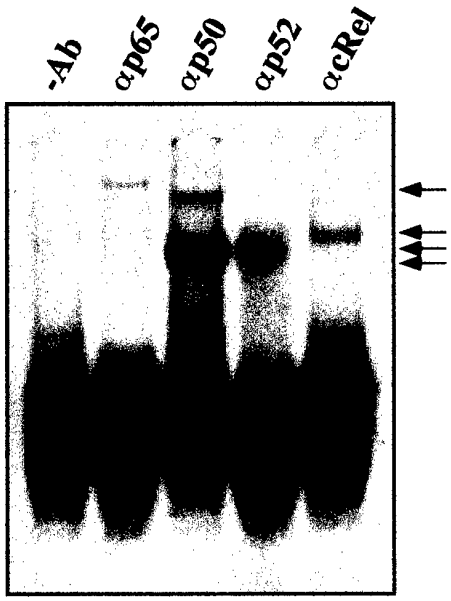
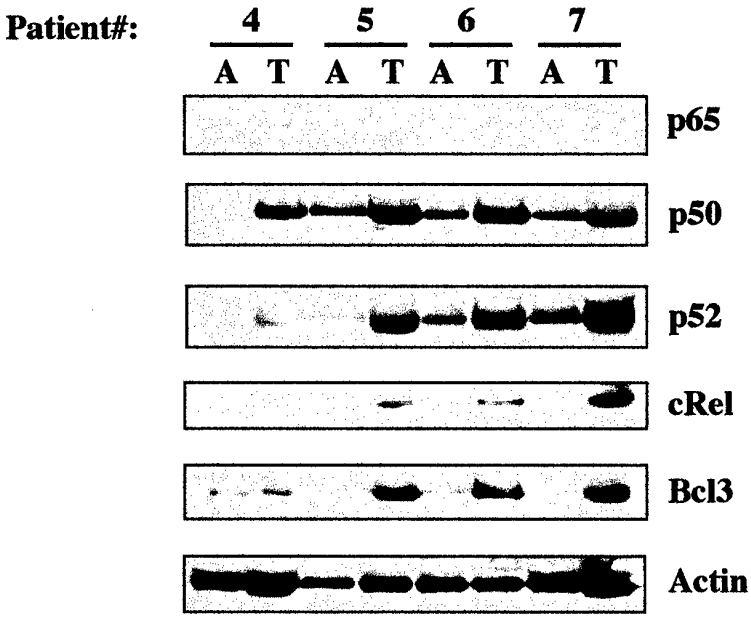


Figure 3



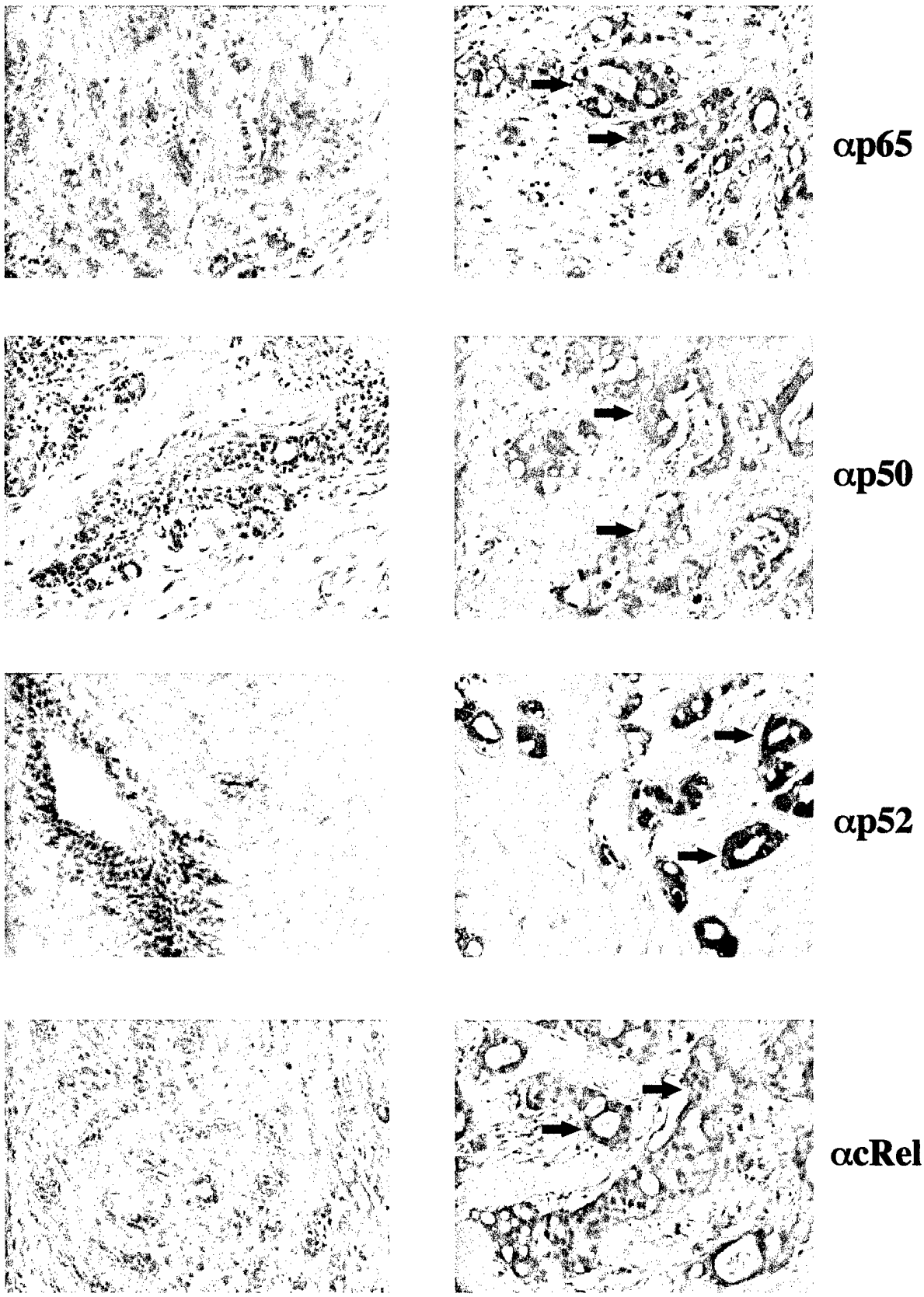
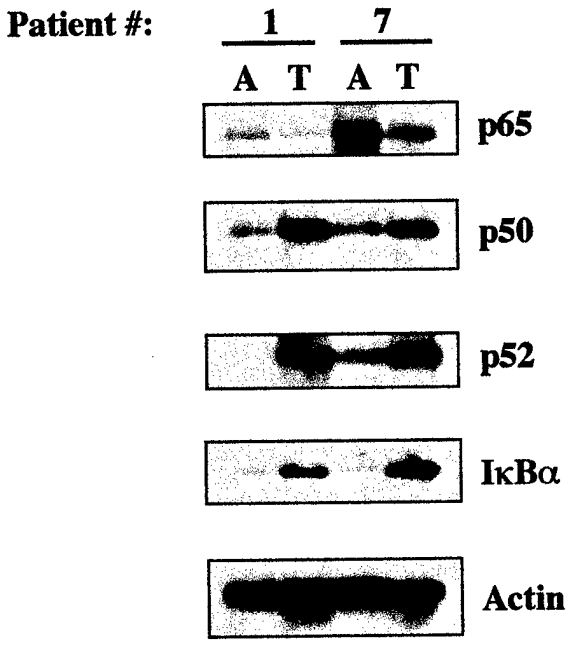

Adjacent
Tumor
αp65
αp50
αp52
αcRel

Figure 5



[CANCER RESEARCH 56, 4773–4777, October 15, 1996]

Internal Sequence Variations in the Ha-*ras* Variable Number Tandem Repeat Rare and Common Alleles Identified by Minisatellite Variant Repeat Polymerase Chain Reaction[1]

Kathleen Conway,[2] Sharon N. Edmiston, Barbara S. Hulka, Peter A. Garrett, and Edison T. Liu

Department of Epidemiology [K.C., B.S.H., P.A.G., E.T.L.], Department of Medicine [E. T. L.], and the Lineberger Comprehensive Cancer Center [K.C., B.S.H., S.N.E., E.T.L.], The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

ABSTRACT

In this report, we describe the sequence allelotyping of the Ha-*ras* variable number tandem repeat (VNTR) region using a minisatellite variant repeat (MVR)-PCR approach. This method permits the rapid identification of internal sequence variations among the VNTR alleles, exploiting the presence of two polymorphic sites within the 28-bp repeat subunits that give rise to four distinct repeat types. Using MVR-PCR, 20 to 25 repeats at the 5′ end of the VNTR can be sequenced rapidly and reliably. MVR typing of the common alleles *a1, a2, a3,* and *a4* shows that the first six repeats at the 5′ end of each allele constitutes an invariant region. Beginning with repeat 7, characteristic "signature" MVR patterns emerge for each common allele. The *a1* and *a2* common alleles were found to consist of specific repeat types 1, 2, and 3, whereas *a3* and *a4* contain an additional repeat type 4 not present in the smaller alleles. MVR typing of rare-length alleles indicates that they are comprised of disorganized sequences, although they usually bear a resemblance to one of the common alleles at the 5′-most end. These results suggest that the rare alleles may be generated from recombination or gene conversion-type events involving the common progenitor alleles. MVR typing could, therefore, improve the ascertainment of rare Ha-*ras* alleles and may provide molecular insights into the genesis of cancer-associated alleles.

INTRODUCTION

A number of carefully conducted studies strongly suggest that rare alleles of the Ha-*ras1* proto-oncogene are associated with increased susceptibility to a variety of cancers (1–10). Although the mechanism underlying this association is unknown, the Ha-*ras* VNTR[3] can function as an enhancer (11, 12), and it has been suggested that the rare Ha-*ras* alleles may bind more avidly to transcription factors (13, 14).

The polymorphism at the Ha-*ras* locus is due to the presence of a VNTR located approximately 1 kb downstream from the proto-oncogene (15). Previous studies examining the association of rare Ha-*ras* alleles with cancer have used Southern blotting, which is limited in its ability to adequately resolve small differences in allele lengths, especially for the larger alleles, and therefore may lead to allelic misclassification. In addition, the designation of alleles as common *versus* rare (1), or common, intermediate, and rare (3, 7) based simply upon population frequencies lacks a defining mechanism.

The subjectivity of Ha-*ras* allele length designations and the inaccuracies inherent in Southern hybridization methodology have led us to investigate other potentially more biologically relevant structural features of the Ha-*ras* VNTR that may better correlate with cancer development. Studies by Jeffreys *et al.* (16), Armour *et al.* (17), and Neil and Jeffreys (18) indicate that minisatellite alleles vary not only in repeat copy number but also in the interspersion pattern of repeat sequences along the VNTR. The Ha-*ras* VNTR sequence derived from the EJ bladder carcinoma cell line (15) reveals G/C polymorphisms at positions 7 and 15 in the 28-bp repeat unit (Table 1). Additional point mutations also occur, but these tend to cluster within the repeats located at the ends of the VNTR, consistent with findings in other minisatellite repeat loci (16).

In this study, we describe a modification of the MVR method originally described by Jeffreys *et al.* (16) to identify sequence variations among Ha-*ras* VNTR alleles. Four repeat-specific primers corresponding to the two G/C polymorphic sites and a common anchored primer flanking the VNTR are used to PCR-amplify fragments the lengths of which define the positions of the repeat type along the VNTR. In effect, an allele-specific sequence polymorphism ladder is generated. Using a nonradioactive modification of MVR for rapid screening of DNA samples, we are able to reliably type at least 20 to 25 repeat units from the 5′ end of the VNTR. This method provides a sequence-based differentiation of the rare from the common alleles.

MATERIALS AND METHODS

The MVR-PCR approach used is similar to the four-state MVR method described by Tamaki *et al.* (19). An upstream primer (Amp L) 5′-GGTTGGGGGAGAGCTAGCAGGGCA-3′ was synthesized from sequences flanking the 5′ end of the VNTR. Four repeat-specific primers were synthesized complementary to the four possible repeat types within the VNTR. These repeat-specific primers span the region of each subunit harboring the G/C polymorphisms (primer 1, 5′-N(20)-GGCGTCCCCTGGAGAGAAGGGC-3′; primer 2, 5′-N(20)-GGCGTCCCCTGGAGAGAAGGGG-3′; primer 3, 5′-N(20)-GGCGTCCCCTGGACAGAAGGGG-3′; and primer 4, 5′-N(20)-GGCGTCCCCTGGACAGAAGGGC-3′, with N(20) = 5′-TCCCGCGTCCATGGCAGCTG-3′). The final 3′ nucleotide for each of these repeat-specific primers was positioned at the first G/C polymorphism (Table 1). To prevent progressive shortening at each PCR cycle because of repeat-specific primers priming internally in PCR products, repeat-type detection and subsequent amplification is uncoupled by adding a 20-nucleotide TAG sequence to the 5′ end of each repeat-specific primer; the primer corresponding to this TAG is 5′-TCCCGCGTCCATGGCAGCTG-3′. Amplification is then carried out with a low concentration of the repeat-specific primer and high concentrations of Amp L and TAG primers.

MVR-PCR was performed in four separate 40-μl reactions containing 5–10 ng of genomic DNA, 1 μM Amp L, 1 μM TAG, 50 mM KCl, 45 mM Tris-HCl (pH 8.8), 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.5 μM EDTA, 110 μg/ml BSA, 2.5 units AmpliTaq polymerase (Perkin-Elmer), 1 mM each deoxyribonucleotide triphosphate, and 10 nM of repeat-specific primer 1, 2, 3, or 4. Reactions were hot-started by adding the deoxynucleotide triphosphates after tubes reached 96°C. Amplification was carried out as a two-step PCR with the following cycle parameters: 1 cycle of 96°C for 9 min, followed by 27 cycles of 1.3 min at 96°C and 6 min at 70°C, with a final extension of 10 min at 70°C. The MVR-PCR product ladders were separated by electrophoresis in 4% Metaphor agarose gels 40 cm in length using 1× TBE buffer (45 mM Tris-borate, pH 8.3, 1 mM EDTA), stained with ethidium bromide followed by destaining, and visualized under UV illumination using a

Received 4/29/96; accepted 8/15/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the University of North Carolina Breast Cancer SPORE Grant P50-CA58223 (to K. C., B. S. H., and E. T. L.) and U. S. Army Grant DAMD17-94-J-4053 (to K. C.). E. T. L. is a Leukemia Society Scholar.

[2] To whom requests for reprints should be addressed, at Department of Epidemiology, University of North Carolina at Chapel Hill, Campus Box 7400, 2101 McGavran-Greenberg, Chapel Hill, NC 27599. Phone: (919) 966-2180; Fax: (919) 962-3405.

[3] The abbreviations used are: VNTR, variable number tandem repeat; MVR, minisatellite variant repeat.

Table 1 *Sequence polymorphisms within the 28 bp subunits of the Ha-ras VNTR*

Polymorphisms are shown at positions 7 and 15 from the 5′ end.

MVR repeat type	Repeat Sequence	No. repeats in EJ cell line
[a]1:	5′-a c a c t c G c c c t t c t C t c c a g g g g a c g c c-3′	12/29
2:	. . . . . C . . . . . C	7/29
3:	. . . . . C . . . . . G	5/29
4:	. . . . . G . . . . . G	1/29

[a] Sequence 1 is the 28 bp consensus sequence present in 12 of the 29 repeats in the EJ VNTR.

film, and MVR codes beginning at the 5′ end of the VNTR were read from the bottom of the gels.

RESULTS

We have adapted MVR technology to decipher sequence variations within the Ha-*ras* VNTR. To verify the specificity of our primers and to be certain that this method accurately identifies the VNTR internal sequence, we first compared the expected Ha-*ras* VNTR sequence with the MVR pattern obtained from the EJ bladder carcinoma cell line, for which the hemizygous VNTR sequence is known (15). The Ha-*ras* VNTR in the EJ line is an *a1*-length allele, and displays the MVR pattern 5′-001321-13113221311222. . . which corresponds to the A1a MVR subtype shown in Table 2. The predicted sequence, 5′-001321-14113221311223. . . , differs from the *A1a* pattern at positions 8 and 12. These changes of a type 4 to type 3 and a type 3 to type 2 repeats, respectively, may represent either errors in the reported sequence or mutations that occurred in our particular EJ clone. The fact that the EJ Ha-*ras* VNTR sequence corresponds exactly to the common *A1a* allele sequence, however, suggests the former possibility.

MVR-PCR of the Ha-*ras* VNTR distinguishes five types of repeats we refer to as 0, 1, 2, 3, and 4. Repeats 1, 2, 3, and 4 correspond to the four possible sequence combinations resulting from the two G/C polymorphisms within the 28-bp subunits (Table 1). The 0 or null repeats, which are seen as blank positions on the gels, contain mutations that prevent primer binding and are, therefore, unamplifiable. Between 20 and 25 repeats could be reliably typed from the 5′ end of the VNTR using this technique.

Using MVR, we proceeded to define the range of sequence allelotypes for the four common-length Ha-*ras* VNTR alleles. All length assignments were derived previously using Southern hybridization methodology (7). Since the *a1* common allele is present at the highest frequency in the population and since homozygous MVR patterns are the simplest to interpret, displaying only a single band at each ladder position, we began the analyses on individuals known to be homozygous for the *a1* allele. A total of 40 *a1/a1* germline DNA samples were evaluated (Table 3). Seventy-eight of 80 *a1* alleles showed one of two common MVR patterns identified as *A1a* or *A1b*. These two *A1* MVR subtypes differ only at positions 17 and 18, as shown in Table 2 and Fig. 1, *A* and *B*.

Once the *a1* pattern was firmly established, we performed MVR typing on *a1* heterozygotes (*a1/a2, a1/a3,* and *a1/a4*) to deduce the patterns of the other common alleles. These sequences were determined by subtracting out the *A1a* or *A1b* pattern from the dual pattern seen in these individuals. This approach was used previously by Jeffreys *et al.* (16) in their pedigree analysis of the MS32 VNTR. The putative *a2, a3,* and *a4* MVR patterns were then verified in homozygotes, although these allelotypes occurred very infrequently (Fig. 1).

Table 2 *MVR patterns of common or rare length Ha-ras VNTR alleles*

		Common alleles							Rare alleles					
		A1a[a]	A1b	A2	A3a	A3b	A3c	A4	R343	R37	R45	R379	R348	R397
	Repeat position	1.00 kb[b] (0.78)[c]	(0.20)	1.45 (1.00)	(0.31)	2.05 (0.35)	(0.23)	2.5 (1.00)	1.175	1.225	1.750	1.750	2.225	2.225
5′	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	1	1	1	1	1	1	1	1	1	1	1	1	1
	4	3	3	3	3	3	3	3	3	3	3	3	3	3
	5	2	2	2	2	2	2	2	2	2	2	2	2	2
	6	1	1	1	1	1	1	1	1	1	1	1	1	1
	7	1	1	2	2	2	2	4	**1** (A1)[e]	1 (?)	**4** (A4)	2 (?)	**2** (A3)	**4** (A4)
	8	3	3	2	1	1	1	1	**3**	1	**1**	3	**1**	**1**
	9	1	1	2	1	1	1	2	**1**	1	**2**	1	**1**	**2**
	10	1	1	2	3	3	3	2	**1**	1	**2**	1	**3**	**2**
	11	3	3	1	2	2	2	4	**3**	3	**4**	3	**2**	2
	12	2	2	3	1	1	1	1	**2**	3	**1**	2	**1**	2
	13	2	2	1	(4)	(1)	(4)	2	**2**	1	**2**	1	2	2
	14	1	1	1	1	1	1	3	**1**	1	**3**	4	1	1
	15	3	3	3	(2)	(2)	(3)	2	2	3	1	2	1	3
	16	1	1	1	2	4	2	4	2	1	3	3	3	1
	17	(1)[d]	(2)	3	4	1	4	1	3	1	3	2	2	1
	18	(2)	(1)	1	1	2	1	2	1	3	4	4	1	1
	19	2	2	2	2	3	2	2	3	1	1	1	2	2
3′	20	2	2	3	3	1	3	2	1	1	2	2	1	4

[a] A, common allele; R, rare allele.
[b] Allele length in kilobase pairs determined by Southern hybridization.
[c] Proportion of each common allele with this MVR pattern.
[d] Variant repeat positions in a1 or a3 alleles are indicated by ( ).
[e] Bolded repeats in the rare alleles R343, R45, R348, and R397 indicate 5′ sequence similarity to the common alleles A1, A3, or A4 as indicated.

Table 3 *MVR sequence variation in the common length Ha-ras alleles a1, a2, a3, and a4*

Length allelotype[a]	No. alleles analyzed (no. persons)	Number of common length alleles with this MVR sequence													
		a1 alleles				a2 alleles		a3 alleles						a4 alleles	
		A1a	A1a-V[b]	A1b	A1b-V	A2	A2-V	A3a	A3a-V	A3b	A3b-V	A3c	A3c-V	A4	A4-V
a1/a1	80 (40)	60	2	18	0	—[c]	—	—	—	—	—	—	—	—	—
a1/a2	80 (40)	36	0	4	0	36	4	—	—	—	—	—	—	—	—
a1/a3	78 (39)	29	1	8	1	—	—	12	1	14	2	9	0	—	—
a1/a4	80 (40)	29	1	10	0	—	—	—	—	—	—	—	—	39	1
Total alleles	328 (164)	154	4	40	1	36	4	12	1	14	2	9	0	39	1
Sequence variants in the common length alleles (%)			5/199 (2.5)			4/40 (10.0)				3/39 (7.7)				1/40 (2.5)	

[a] Allele length determined by Southern blotting methods.
[b] -V, sequence variants displaying one or two repeat differences within the first 20 repeats at the 5′ end as compared with the known common sequence(s).
[c] —; none found.

The MVR sequences (repeats 1–20) for the common-length alleles are given in Table 2. The MVR pattern (5′-001321. . . 3′) over the first six repeats at the 5′ end of all alleles was exactly the same. Because the first two repeats are 0 repeats, the first readable repeat is repeat position 3, seen as a 155-bp fragment at the bottom of the gels (Fig. 1, *A* and *B*). Beginning with repeat position 7, each common-length allele displays a characteristic "signature" sequence. One MVR sequence was identified for each of the common alleles *a2* and *a4*, whereas two MVR subtypes were identified for *a1* (*A1a* and *A1b*) and three were found for *a3* (*A3a, A3b,* and *A3c*), each differing by only one or two repeats from the other at the positions indicated in Table 2. Of all *a1*-length alleles analyzed, 97.5% had the exact *A1a* (5′-001321-13113221311222. . .; 77.4%) or *A1b* (5′-0013211311322-1312122. . . ; 20.1%) sequence over the first 20 repeats (Table 3). Of *a2*-length alleles, 90% displayed the exact A2 MVR sequence (5′-001321-22221311313123). For *a3*-length alleles, 92.3% were of the A3a (5′-001321-21132141224123. . . ; 30.8%), *A3b* (5′-001321211-32111241231. . . ; 38.5%), or *A3c* (5′-001321-21132141324123. . . ; 23.1%) subtypes, whereas 97.5% of *a4*-length alleles had the *A4* sequence (5′-001321-41224123241222. . .). Interestingly, the *a1* and *a2* alleles are composed solely of three specific 28-bp repeat types (types 1, 2, and 3; Table 2), whereas *a3* and *a4* are characterized by the presence of the type 4 repeat not found in *a1* and *a2*.

The remainder of the common-length alleles were minor variants (denoted by -V) of the MVR sequence subtypes described above. These variants, shown in Table 4, were characterized as having one or two repeat alterations occurring as additions, deletions, or changes in repeat types in otherwise common MVR sequences. The frequencies of variation from the established MVR patterns were 2.5% for *a1*-length alleles, 10% for *a2* alleles, 7.7% for *a3* alleles, and 2.5% for *a4* alleles (Table 3). All of the MVR structural variants identified involved intact 28-bp subunits. We did not detect any Ha-*ras* VNTR alleles containing repeat units of abnormal length (either shorter or longer than the expected 28 bp), which would place the MVR ladders of the two constituent alleles out of register.

In contrast to the common-length alleles that have characteristic and relatively stable sequences, MVR typing of six germline DNA samples previously shown to have rare-length alleles by Southern analysis indicated that these alleles possessed disorganized internal sequences. The sequence alterations involved more than two repeats, and for some rare alleles, involved the entire 5′ sequence. The rare alleles *R343, R45, R348,* and *R397,* shown in Table 2 and Fig. 1*C*, appear to be derived at the 5′ end from the common alleles *A1, A3,* or *A4*. The *R348* rare allele (5′-001321-21132121132121. . .) possesses a sequence identical to *A3* through repeat 12. However, beginning with repeat 13, the *A3* pattern is disrupted. Similarly, the *R45* (5′-001321-41222221311124. . .) and *R397* (5′-001321-41222221311124. . .) rare alleles begin as

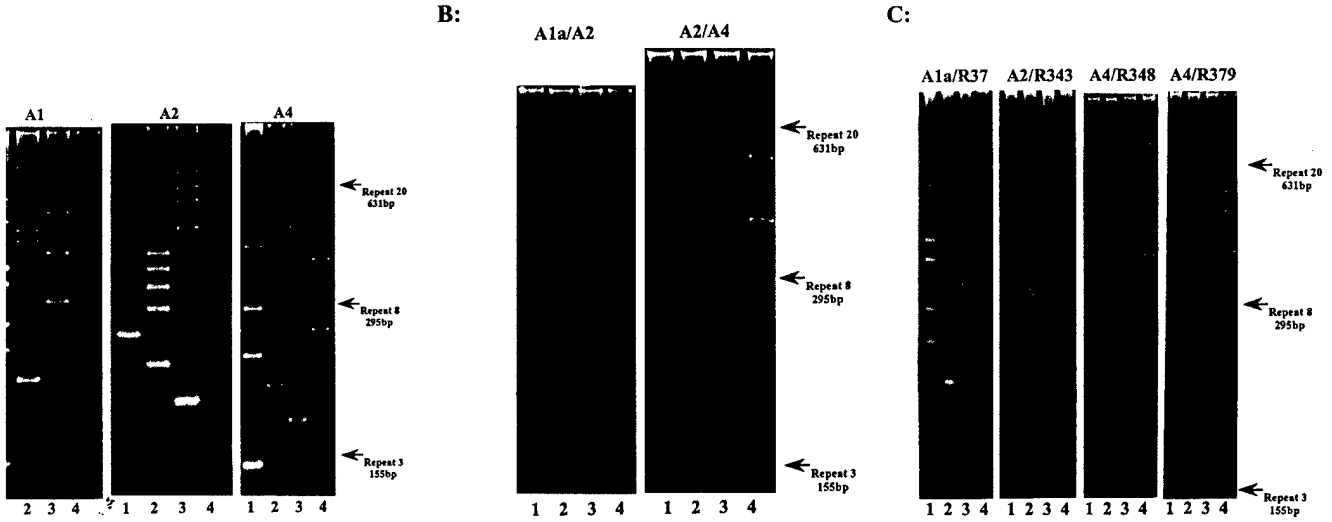


Fig. 1. Examples of Ha-*ras* VNTR MVR patterns from individuals with common or rare alleles. MVR-PCR was performed using primers specific for the four repeat types corresponding to the G/C polymorphisms identified from the EJ bladder carcinoma VNTR sequence. *A*, MVR patterns of individuals homozygous for the common-length alleles *a1, a2,* or *a4*. *B*, MVR patterns of heterozygous individuals with *a1/a2* or *a2/a4* common-length alleles. *C*, MVR patterns of heterozygous subjects with one common-length and one rare-length allele. The heterozygous MVR allelotypes shown are *A1a/R37, A2/343, A4/348,* and *A4/379*. The first readable repeat for each subject is the 155-bp fragment corresponding to repeat 3 at the bottom of each gel.

Table 4 *Variants of the Ha-ras VNTR common allele MVR sequences*

	Repeat position	Ala-Var				A1b-Var	A2-Var				A3a-Var	A3b-Var		A4-Var
		V75	V178	V214	V525	V128	V139	V270	V283	V325	V248	V135	V581	V33
5′	1	0	0	0	0	0	0[a]	0	0	0	0	0	0	0
	2	0	0	0	0	0	1	0	0	0	0	0	0	0
	3	1	1	1	1	1	3	1	1	1	1	1	1	1
	4	3	3	3	3	3	2	3	3	3	3	3	3	3
	5	2	2	2	2	2	1	2	2	2	2	2	2	2
	6	1	1	1	1	1	2	1	1	1	1	1	1	1
	7	1	1	1	1	1	2	2	2	(1)	2	2	2	4
	8	(2)	3	3	3	3	2	2	2	2	1	1	1	1
	9	3	1	1	1	1	2	2	2	2	1	1	1	2
	10	1	1	1	1	1	1	2	2	2	3	3	3	2
	11	1	2	3	3	3	3	1	1	1	2	2	2	4
	12	3	2	2	2	2	(3)	3	3	3	1	1	1	1
	13	2	1	2	2	2	1	1	1	1	1	1	1	2
	14	2	3	(2)	1	1	1	1	1	1	2	(2)	1	3
	15	1	1	1	3	3	3	3	3	3	2	2	2	2
	16	3	1	3	1	1	1	1	1	1	4	4	4	4
	17	1	2	1	1	2	3	3	3	3	1	1	1	1
	18	1	2	1	2	1	1	1	1	1	2	2	(1)	2
	19	2	2	2	2	2	2	2	(1)	2	3	3	3	2
3′	20	2	3	2	(3)	(3)	3	(2)	2	3	2	1	1	(1)

[a] Variant repeat positions in the common MVR sequences are indicated by ( ) for a change in repeat type or insertion of a repeat or _ for deletion of the following repeat.

A4 alleles, but again the *A4* sequences are lost farther downstream. Moreover, the *R37* (5′-001321-11113311311311. . .) and *R379* (5′-001321-23113214232412. . .) rare alleles bear little resemblance to the common alleles.

This MVR method permits us to read the sequence of approximately 25 repeats from the 5′ end of the VNTR, so that nearly the complete MVR sequence of the *a1* common allele can be characterized. However, only about 35% of the *a4* common allele can be determined. Despite this inherent bias toward sequence ascertainment of the smaller alleles, we were still able to uncover significant sequence variation for large alleles known to have rare lengths.

DISCUSSION

A number of recent studies have focused on the putative role of Ha-*ras* rare alleles as markers of genetic susceptibility to a variety of cancers, including cancers of the breast, lung, and bladder (1–10). In this report, we have described an adaptation of the MVR technique for sequence allelotyping of the Ha-*ras* VNTR. Our preliminary analyses of the 5′ end of the VNTR indicate that Ha-*ras* allele length is closely linked with allele sequence for the common alleles *a1, a2, a3,* and *a4* in that each common-length allele possesses its own characteristic sequence(s). For example, nearly all *a2*-length alleles possess the same identical 5′ MVR sequence. In contrast, MVR typing of six rare-length alleles suggests that these alleles are composed of disorganized internal sequences, although they appear to be derived in most cases from common progenitor alleles. A previous report by Kasperczyk *et al.* (20) suggested that rare alleles are derived from the common allele nearest in size. Based upon MVR sequence typing, this does not appear to be true for the rare allele *R45*. The *R45* allele lies between *a2* and *a3* in length, yet possesses an *a4*-like MVR sequence at the 5′ end. Thus, this rare allele appears to be derived from an *a4* allele rather than *a2* or *a3*.

Jeffreys and coworkers (16–19) have extensively characterized a number of other VNTRs, including MS32 (16, 19), MS205 (17), and MS31A (18), using the MVR technique. These VNTRs, which are of the class used for DNA fingerprinting, have polymorphic frequencies of 95% or more based on length differences alone and by MVR exhibit even higher levels of allelic variation. In contrast, the Ha-*ras* VNTR has a much lower level of population heterozygosity (approximately 65% by length, as determined by Southern hybridization; Ref. 21), and the common Ha-*ras* alleles appear to be relatively stable, maintaining a strict association between length and sequence.

The rare Ha-*ras* VNTR alleles, however, appear to have resulted from recombinatory events, as evidenced by the resemblance to common allele MVR patterns at the 5′ end of the VNTR but with significant rearrangement of the internal repeats. Jeffreys *et al.* (22) have suggested that interallelic recombination or gene conversion-like events, possibly occurring during meiosis, are responsible for the allelic variation at the *MS32* and *MS31A* loci (22), as evidenced by the preservation of VNTR flanking regions but rearrangement of internal sequences. Furthermore, the mutations occurring in certain minisatellites are polar in that they take place preferentially at one end of the tandem repeat array, and this instability may be controlled by elements outside the minisatellite (16, 22, 23).

Our data raise the possibility that rare alleles are a reflection of an inherent genetic instability, which is the direct effector of cancer susceptibility. MVRs, therefore, can be used for the rapid and precise identification of the rare Ha-*ras* variants within a population. In addition, the analysis of repeat architecture at the Ha-*ras* locus may provide clues to the mechanism of cancer association with the rare alleles.

REFERENCES

1. Krontiris, T. G., DiMartino, N. A., Colb, M., and Parkinson, D. R. Unique allelic restriction fragments of the human Ha-*ras* locus in leukocyte and tumour DNAs of cancer patients. Nature (Lond.) *313:* 369–374, 1985.
2. Krontiris, T. G., Devlin, B., Karp, D. D., Robert, N. J., and Risch, N. An association between the risk of cancer and mutations in the *HRAS1* minisatellite locus. N. Engl J. Med., *329:* 517–523, 1993.
3. Krontiris, T. G., DiMartino, N. A., Mitcheson, H. D., Lonergan, J. A., Begg, C., and Parkinson, D. R. Human hypervariable sequences in risk assessment: rare Ha-*ras* alleles in cancer patients. Environ. Health Perspect., *76:* 147–153, 1987.
4. Barkardottir, R. B., Johannsson, O. T., Arason, A., Gudnason, V., and Egilsson, V. Polymorphism of the c-Ha-*ras-1* proto-oncogene in sporadic and familial breast cancer. Int. J. Cancer, *44:* 251–255, 1989.
5. Boehm, T. L. J., Hirth, H-P., Kornhuber, B., and Drahovsky, D. Oncogene amplifications, rearrangements and restriction fragments length polymorphisms in human leukaemia. Eur. J. Clin. Oncol., *23:* 623–629, 1987.
6. Corell, B., and Zoll, B. Comparison between the allelic frequency distribution of the Ha-*ras1* locus in normal individuals and patients with lymphoma, breast and ovarian cancer. Hum. Genet., *79:* 255–259, 1988.
7. Garrett, P. A., Hulka, B. S., Kim, Y. L., and Farber, R. A. *HRAS* protooncogene polymorphism and breast cancer. Cancer Epidemiol., Biomarkers & Prev., *2:* 131–138, 1993.
8. Lidereau, R., Escot, C., Theillet, C., Champeme, M-H., Brunet, M., Gest, J., and

Callahan, R. High frequency of rare alleles of the human c-Ha-*ras-1* proto-oncogene in breast cancer patients. J. Natl. Cancer Inst., *77:* 697–701, 1986.

9. Sheng, Z. M., Martine, G., Gabillet, M., Spielman, M., and Riou, G. c-Ha-*ras-1* polymorphism in breast cancer patients and a control population. Oncogene Res., *2:* 245–250, 1988.
10. Sugimura, H., Caporaso, N. E., Modali, R. V., Hoover, R. N., Resau, J. H., Trump, B. F., Lonergan, J. A., Krontiris, T. G., Mann, D. L., Weston, A., and Harris, C. C. Association of rare alleles of the Harvey *ras* protooncogene locus with lung cancer. Cancer Res., *50:* 1857–1862, 1990.
11. Cohen, J. B., Walter, M. V., and Levinson, A. D. A repetitive sequence element 3′ of the human c-Ha-*ras1* gene has enhancer activity. J. Cell. Physiol., *5* (Suppl.)*:* 75–81, 1987.
12. Spandidos, D. A., and Holmes, L. Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-*ras1* gene. FEBS Lett., *218:* 41–46, 1987.
13. Trepicchio, W. L., and Krontiris, T. G. Members of the *rel*/NK-B family of transcriptional regulatory proteins bind the *HRAS1* minisatellite DNA sequence. Nucleic Acids Res., *20:* 2427–2434, 1992.
14. Green, M., and Krontiris, T. G. Allelic variation of reporter gene activation by the *HRAS1* minisatellite. Genomics, *17:* 429–434, 1993.
15. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Goeddel, D. V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature (Lond.) *302:* 33–37, 1983.
16. Jeffreys, A. J., MacLeod, A., Tamaki, K., Neil, D. L., and Monckton, D. G. Minisatellite repeat coding as a digital approach to DNA typing. Nature (Lond.) *354:* 204–209, 1991.
17. Armour, J. A. L., Harris, P. C., and Jeffreys, A. J. Allelic variation at minisatellite *MS205* (*D16S309*): evidence for polarized variability. Hum. Mol. Genet., *2:* 1137–1145, 1993.
18. Neil, D. L., and Jeffreys, A. J. Digital DNA typing at a second hypervariable locus by minisatellite variant repeat mapping. Hum. Mol. Genet., *2:* 1129–1135, 1993.
19. Tamaki, K., Monckton, D. G., MacLeod, A., Allen, M., and Jeffreys, A. J. Four state MVR-PCR: increased discrimination of digital DNA typing by simultaneous analysis of two polymorphic sites within minisatellite variant repeats at *D1S8*. Hum. Mol. Genet., *2:* 1629–1632, 1993.
20. Kasperczyk, A., DiMarino, N. A., and Krontiris, T. G. Minisatellite allele diversification: the origin of rare *HRAS1* alleles. Am. J. Hum. Genet., *47:* 854–859, 1990.
21. Krontiris, T. G. Minisatellites and human disease. Science (Washington DC), *269:* 1682–1683, 1995.
22. Jeffreys, A. J., Tamaki, K., MacLeod, A., Monckton, D. G., Neil, D. L., and Armour, J. A. L. Complex gene conversion events in germline mutation at human minisatellites. Nat. Genet., *6:* 136–145, 1994.
23. Monckton, D. G., Neumann, R., Guram, T., Tretwell, N., Tamaki, K., MacLeod, A., and Jeffreys, A. J. Minisatellite mutation rate variation associated with a flanking DNA sequence polymorphism. Nat. Genet., *8:* 162–170, 1994.

Allelotyping of the H-ras Variable Number Tandem Repeat (VNTR) in a Breast Cancer Case-Control Study

Kathleen Conway[1,2], Sharon N. Edmiston[2], Scott Joens[1],
Barbara S. Hulka[1,2], Peter A. Garrett and Edison T. Liu[3]

Department of Epidemiology[1] and
the Lineberger Comprehensive Cancer Center[2],
The University of North Carolina at Chapel Hill,
and the
Division of Clinical Sciences of the National Cancer Institute[3]

Running Title: The H-ras VNTR in breast cancer susceptibility

Key Words: H-ras, VNTR, rare alleles, PCR, breast cancer

Abbreviations Used:
VNTR: variable number tandem repeat
MVR; minisatellite variant repeat
PCR; polymerase chain reaction
OR; odds ratio

Address requests for reprints to:
Kathleen Conway
Department of Epidemiology
University of North Carolina at Chapel Hill
Campus Box 7400
2101 McGavran-Greenberg Hall
Chapel Hill, North Carolina 27599
Telephone (919) 966-2180
FAX (919) 962-3405

ABSTRACT

Previous studies exploring the association of rare alleles of the H-ras variable number tandem repeat (VNTR) with cancer have employed Southern hybridization methods which do not adequately resolve alleles differing by only a few 28 bp repeats. We have recently developed a polymerase chain reaction (PCR) assay to determine H-ras VNTR length, and a minisatellite variant repeat (MVR)-PCR method to identify VNTR sequence variations. These methods were applied to a case-control study of breast cancer which had previously been screened for H-ras polymorphisms using Southern hybridization. The purpose of the present work was to determine whether the more refined PCR-based H-ras allelotyping methods influence the identification of rare alleles, and subsequently the risk of breast cancer associated with rare alleles. Our results demonstrate that the PCR length assay permits resolution of single repeat differences in H-ras VNTR allele length regardless of allele size. MVR-PCR, together with allele length analysis, further improves the detection of structurally-altered rare alleles and permits the determination of the parental origin of rare alleles. H-ras allelotyping indicated that African-American and white control populations differed in their normal spectrum of H-ras alleles. Rare alleles were significantly associated with breast cancer in African-American women (OR=5.6, 95% CI=1.2-25.8), but not in white women (ORs ranged from 0.8 to 1.3), although rare alleles with lengths of 59 repeats occurred more frequently in white women with breast cancer. These findings are discussed with respect to H-ras allele classification and putative functions of the H-ras VNTR.

INTRODUCTION

The H-ras protooncogene locus displays a DNA polymorphism which is due to a variable number tandem repeat (VNTR) sequence located 1 kilobase (kb) downstream from the coding region of the gene (1). Variation in the number of 28 base pair (bp) repeats results in a series of alleles detectable by restriction enzyme digestion and Southern blot analysis. The polymorphic H-ras minisatellite consists of four common progenitor alleles and at least several dozen rare alleles that are presumably derived by mutation of the common alleles. Krontiris et al (2) originally reported that individuals with certain types of cancer possessed significantly more rare H-ras alleles as compared with normal unaffected controls. Numerous studies have subsequently attempted to confirm the association of rare alleles with cancer, but have yielded conflicting results which may be due in part to small sample sizes or technical differences between laboratories (3-11). All previous studies evaluating cancer risk associated with the H-ras VNTR have utilized Southern hybridization methods. Clearly, the restriction enzymes and electrophoretic conditions employed in Southern hybridization may vary, and large alleles varying by only 28 bp in length may not be adequately resolved by this method.

A meta-analysis conducted by Krontiris et al (12) of studies using Southern hybridization technology upheld the association of H-ras rare alleles with certain cancers. This study suggested that despite the weak risk imposed by a rare H-ras allele, the frequency of these alleles in the general population indicates they may be involved in 1 in 11 cases of breast, bladder or colorectal cancer. A second recent meta-analysis by Weston and Godbold (13) focusing on breast and lung cancer reviewed many of the same studies and reported similar findings. Given the moderate cancer risk that appears to be associated with some H-ras alleles, more refined methods and specific criteria are needed to define these "rare" high risk alleles. Ideally, high risk alleles should be identified based upon structural or possibly even functional characteristics of the H-ras VNTR, in addition to population frequencies.

We have previously demonstrated that the 28 bp subunits of the H-ras VNTR carry G/C polymorphisms at two positions, yielding four different repeat types; these sequence variations

can be identified using a modification of the minisatellite variant repeat (MVR) method which permits the partial sequence analysis of 28 bp tandemly repeated subunits inward from the 5' end of the H-ras VNTR (14). More recently, we have also developed a PCR assay to detect allele length, and have used this in combination with MVR to more fully characterize H-ras VNTR structure.

The purpose of the present study was to determine whether the more refined PCR allelotyping techniques influence the detection, classification, and subsequently, the risk of breast cancer associated with a rare allele.[7] To this end, we have applied the PCR methods to a case-control study of breast cancer previously analyzed for H-ras allele length polymorphisms by Southern blotting (15), enabling us to validate the PCR approach and to ensure that PCR detects all H-ras alleles, even the large alleles which amplify less efficiently. The PCR length assay has proven to be extremely reliable and can detect single repeat unit differences in allele length among even the largest alleles, while the 5' MVR method permits identification of allelic sequence rearrangements and in most cases the parental origin of rare alleles. These methods allow H-ras allelotyping on minute quantities of DNA and when used in combination, they improve the detection of rare or structurally-altered alleles.

MATERIALS AND METHODS

Subjects The subjects analyzed in this report are part of a case-control study of breast cancer previously evaluated for H-ras polymorphisms using restriction enzyme digestion with Msp I/Hpa II, followed by Southern blotting (15). For the present study, we have utilized a subset of the original case-control population. The cases include only incident cases of invasive breast cancer seen at the UNC Hospital Breast Clinic, the Duke University Multidisciplinary Breast Clinic or the Durham Clinic. The controls were women who were seen at two general medicine clinics at UNC Hospital for health problems unrelated to cancer or the breast. Cases and controls were age-matched to within 5 years. All cases or controls considered to be of a racial group other than African-American or white have been eliminated from the present study.

Germline DNA isolated from peripheral blood leukocytes was available from the existing subject groups (15).

PCR Allele Length Analyses For H-ras allele length typing, PCR amplification was performed using 125 ng germline DNA in a 50 µl reaction consisting of 50 mM Tris-HCl, pH 9.2, 14 mM ammonium sulfate, 1.75 mM $MgCl_2$, 300 nM each primer, 5'-GCTCCTGGCCTCGGGAAGTCTAT-3' and 5'-AGAGCTAGCAGGGCATGCCGCT-3' (16), 350 µM of each deoxynucleotide triphosphate (dNTP), and 0.75 units Expand Long Template PCR enzyme (Boehringer Mannheim). Reactions were carried out under cycle parameters of: 1 cycle of 94°C for 7 min., followed by 30 cycles of 1 min. at 94°C and 6 min. at 68°C, followed by a final extension of 10 min. at 68°C. All PCR reactions were hot-started by adding the dNTPs separately during the first cycle of 94°C. Negative controls without DNA were included in each PCR run. Each 20 µl aliquot of PCR product was mixed with 2 µl 6X loading buffer (0.25% bromophenol blue/30% glycerol) and was subjected to electrophoresis in a 1.2 % agarose gel 40 cm in length using TBE buffer (45 mM Tris-borate, pH 8.3 and 2 mM EDTA). The molecular size marker VII (Boehringer Mannheim) and a 123 bp ladder (Gibco-BRL) were run in approximately every seventh and eighth lane on all gels to minimize artifacts due to gel distortion. Gels were stained with ethidium bromide and destained in water to allow direct visualization of the alleles. PCR products were first run to determine approximate allele sizes, then were re-run together in groups of similar size for longer times to achieve more refined sizing of specific alleles. In this way, small differences in allele lengths could be easily detected. When this preliminary allele length typing was complete, all alleles of each length (for example, all alleles thought to be 85 repeats, etc.) were run again and compared in order to ensure consistency of length assignments across the entire data set. Since the approximate allele sizes were already known from the previous Southern hybridization studies, the ability of the PCR to amplify large alleles, particularly in heterozygous samples, could also be directly monitored.

5' MVR Sequence Analyses The sequence analyses of the H-ras VNTR were performed on germline DNA using MVR-PCR as previously described (14).

RESULTS

H-ras Allele Length Typing by PCR H-ras VNTR alleles were characterized in 157 incident breast cancer cases and 198 unaffected controls, using a combination of PCR allele length and 5' MVR sequence methods. All subjects were first evaluated for H-ras allele length polymorphisms using the PCR approach; an example of PCR amplification of the H-ras VNTR is shown in Figure 1, and a summary of allele lengths is given in Table 1. Southern blotting identified four common alleles within this population, a1 (1.00 kb), a2 (1.45 kb), a3 (2.05 kb) and a4 (2.50 kb). PCR demonstrated that there was a cluster of alleles, differing in length by one or a few repeats, around each common allele mode. The modal common allele lengths identified by PCR were 30 repeats for a1, 46 repeats for a2, and 69 repeats for a3. Several alleles ranging from 85 to 88 repeats were identified for a4, with the 87 repeat allele occurring most frequently.

In order to more directly compare the allelic resolution achieved with PCR versus Southern blotting, we determined the number of alleles judged to be a1, a2, a3 or a4 by each method. Of 399 alleles identified as a1 by Southern blotting, 29 (7.3%) had a length other than 30 repeats by PCR. Of 84 a2 alleles identified by Southern blotting, 12 (14.3%) had a length other than 46 repeats. Greater variation in allele length was observed around the larger a3 and a4 alleles. Of 62 a3 alleles identified by Southern blotting, 13 (21.6%) had a length other than 69 repeats. Of 69 a4 alleles detected by Southern blotting, 35 (50.7%) had a length other than 87 repeats.

Because the PCR approach to H-ras allelotyping provides increased resolution over Southern blotting, it was of interest to determine whether this would affect allele length classification and result in a re-distribution of alleles from the rare to the common category, or vice versa. As shown in Table 1, we designated rare alleles as those having individual frequencies of $\leq$0.5% in the combined control group (blacks and whites), according to the cut point utilized in the original Southern blotting study by Garrett et al (15), as well as prior studies by Krontiris and coworkers (17). As indicated in Table 1 and shown in the frequency

distribution of Figure 2, the alleles with individual frequencies >0.5% were clustered around the common allele modes.

Racial Variation in H-ras Allele Length Distribution The allele length distributions among the African-American (18 cases, 24 controls) and white (139 cases, 174 controls) subjects are shown in Figure 2 (and also in Table 1). Several trends are evident from this distribution. The white control population displays the expected common alleles of a1 (30 repeats; 57.8% of alleles), a2 (46 repeats; 10.9%), a3 (69 repeats; 7.8%) and a4 (combining the two most common lengths of 87 and 88 repeats; 9.2%). However, the allele length distribution clearly differs among African-American control women. The a1 (30 repeats; 18.8%) and a4 (85 and 88 repeats; 29.2%) alleles are the most common alleles in the black controls while the a2 and a3 common alleles occur very infrequently. The black control group exhibits two additional common alleles, a1+6 (36 repeats; 12.5%) and a1-2 (28 repeats; 14.6%). The two racial groups also exhibit some variation in a4 allele sizes; whites have a4 alleles of precisely 87 and 88 repeats (6.6 and 2.6%, respectively), while blacks possess a4 alleles of 85 and 88 repeats (12.5 and 16.7%, respectively). Fewer a4 alleles but many more rare alleles were noted in the African-American cases compared with the controls, suggesting the possibility that the rare alleles in this population might be derived primarily from a4.

Common H-ras Allele Sequences by 5' MVR The H-ras VNTR 5' sequence patterns identified by the MVR method are listed in Table 2. While the designations a1, a2, a3 or a4 denote allele lengths, A1a, A1.4, A2, A3a or A4, etc., refer to MVR pattern or sequence. We have previously shown that allele length is tightly linked with MVR sequence for each of the H-ras common alleles such that each of these alleles possesses its own characteristic, distinguishing 5' MVR sequence (14). The alleles with A1a and A1b MVR patterns are usually 30 repeats in length. Of 325 alleles with A1a MVR patterns, 310 (95.4%) have a length of 30 repeats. Of 68 alleles with the A1b pattern, 59 (86.8%) have a length of 30 repeats. Similarly, of 81 alleles with the A2 MVR pattern, 68 (84.0%) are 46 repeats in length. Three A3 patterns, A3a, A3b and A3c, (40/57 or 70.2%) are associated with the a3 common allele (69 repeats), while one A4 pattern

was found in alleles making up the a4 group (85-88 repeats) (72/75 or 96.0%). Four other distinct MVR patterns were also identified for alleles previously classified by Southern blotting as intermediate length alleles; these are the a1-2 (28 repeats) length allele having the A1.02 MVR pattern, the a1+2 (32 repeats) allele having the A1.2 pattern, the a1+4 (34 repeats) allele having the A1.4 pattern and the a1+6 (36 repeats) allele having the A1.6 pattern. The fact that each of these H-ras alleles has a specific length associated with a specific sequence suggests these alleles are relatively stable.

Variant and Rare H-ras Allele Sequences by 5' MVR MVR also identified a series of sequence variants that differed from the common progenitor allele pattern by 1 or 2 repeats out of 20 (Table 2). These variant alleles show single repeat insertions, deletions or changes in repeat type, and in the shorter a1 variants, the exact point of repeat insertion or deletion can often be identified. For example, MVR patterns A1aI-1 and A1aI-2 are single repeat insertion variants, and have a type 2 repeat inserted at positions 8 and 14, respectively. Both of these alleles are one repeat longer than the common a1 allele (31 repeats compared with 30). Similarly, for deletion variant A1aD-1, a type 3 repeat has been lost from position 11 compared with the parental A1a allele. MVR variants were found in 24 of 314 (7.6%) breast cancer cases and 23 of 396 (5.8%) controls (OR=1.4; 95% CI=0.8-2.5).

We have previously shown that many rare alleles possess disorganized 5' sequences, suggesting their derivation by some recombinatory mechanism (14). Using 5' MVR criteria, we have defined the rare alleles as those varying in sequence by more than 2 repeats out of 20 compared with the common progenitor allele (Table 2). Eleven unique rare allele patterns were identified by 5' MVR: A1R-1, A1R-2, A1.8, A59-1, A59-2, A59-3, A3R-1, A4R-1, A4R-3, U-1 and U-2.

The specific structure of each rare allele is further described in Table 3. From the 5' MVR sequence, it is clear that most rare alleles are derived from one of the common alleles; hence, the 5' MVR pattern name incorporates the common progenitor allele from which the rare allele is derived, if this can be determined. For rare alleles possessing rare MVR patterns, the

portion of the sequence which deviates from the common allele, or is unique, is underlined. For example, the A4R-1 rare allele is derived from A4 which apparently underwent rearrangement beginning at repeat position 15 from the 5' end of the VNTR. In contrast, rare alleles U-1 and U-2 are unique in both length and sequence and bear no recognizable similarity to any common allele. The rare alleles of the A59 series (A59-1, A59-2, A59-3) all possess the exact same length of 59 repeats, have similar sequences among themselves, but do not resemble any common allele at the 5'-most end. The parental origin of the A59 series or U-1/U-2 alleles cannot be directly established due to their lack of obvious resemblance to any common allele.

Structural Characterization of Rare Alleles by Length and Sequence In order to optimally identify rare and common H-ras alleles, we have combined the structural criteria of allele length and 5'-MVR sequence, and this relationship is illustrated in Table 3. The common alleles have a common MVR sequence matched with the expected common allele length (indicated as CL+CS). The rare alleles can potentially be defined using a number of criteria. They may be rare by length only (RL+CS), by MVR sequence only (CL+RS), by alterations in both length and sequence (RL+RS), or they may possess a mismatched common length and a common sequence (mismatched CL+CS). One example of the mismatched-type rare allele is found in subject 544; this allele has the common a1 length of 30 repeats, but possesses a common A3b MVR pattern, suggesting the progenitor a3 allele underwent rearrangement/truncation to produce an allele of the a1 length. Overall, about half of the rare length alleles in our population also have rare sequences because MVR provides only a limited analysis at the 5' end of the VNTR and does not detect alterations at the 3' end of the longer alleles. Despite this, MVR complemented the length analysis and led to an increased detection of rare alleles (by about 25%) by enabling us to identify structurally abnormal alleles with common lengths (the CL+RS or mismatched CL+CS).

Rare H-ras Alleles and Breast Cancer Risk We determined whether the risk of breast cancer associated with rare H-ras alleles was affected by the length and sequence criteria used to define rarity (Table 4). H-ras alleles were defined as rare if they possessed (1) rare lengths

(RL+CS and RL+RS), or (2) rare MVR sequences (CL+RS and RL+RS), or (3) both rare lengths and rare sequences (RL+RS), or (4) any of these structural characteristics including mismatched CL+CS (all categories combined). As shown in Table 4, the breast cancer risk associated with a rare H-ras allele was substantially elevated in black breast cancer patients, and this result was significant when rarity was defined by length alone, or by any criteria (OR=5.6, 95% CI=1.2-25.8). However, there was not a statistically significant increase in breast cancer risk found in whites using any of the rare allele classification approaches (ORs ranged from 0.8 to 1.3). Although the PCR-based methods identified additional rare alleles not previously detected by Southern hybridization (38 versus 30 rare alleles, respectively), these rare alleles were equally distributed among cases and controls so that the overall breast cancer risk was only minimally affected.

We also determined whether a specific subset of rare alleles might be associated with breast cancer in whites or in African-Americans. Two structural subsets of rare alleles were of particular interest, those derived from the a4 common allele, and rare alleles with lengths of 59 repeats (many of which had an A59-type MVR pattern). Because the allele frequency distribution in Figure 2 demonstrated that the black case group possessed many more rare alleles (44.4%) but fewer a4 common alleles (9.2%) than the corresponding black controls (8.3% for rare and 29.2% for a4 common), we determined if a4-derived rare alleles might carry a higher risk for breast cancer than other rare alleles in this population. The a4-derived rare alleles had either the A4R-1 MVR pattern or were otherwise structurally related to a4 at the 5' end. In blacks, a4-derived rare alleles did not confer a greater breast cancer risk (Fisher's exact test, $p \leq 0.32$ for a4-derived rare) than that due to all rare alleles. The breast cancer risk associated with rare alleles of 59 repeats in length was elevated in whites (OR=5.3, 95% CI=0.5-67.7), but this finding was not statistically significant due to the small numbers of these alleles.

DISCUSSION

The purpose of the present study was to determine whether the more accurate PCR-based methods for H-ras allelotyping influence the detection and classification of rare alleles, and subsequently, the risk of breast cancer associated with these alleles. We have used a combination of 5'-MVR sequence and PCR allele length typing to characterize H-ras VNTR structure in subjects of a breast cancer case-control study for whom H-ras allelotyping had previously been performed using Southern hybridization (15). This approach has enabled us to validate the reliability and sensitivity of the PCR methods.

The PCR length assay accurately detected all H-ras alleles, even in heterozygotes, and it was sufficiently sensitive to permit resolution of single repeat unit differences among the large alleles. PCR enabled us to identify additional alleles that were unresolvable by Southern hybridization, particularly those clustered around the common allele modes. The MVR approach, although limited to sequence analyses at the 5' end of the VNTR, permitted identification of allelic sequence rearrangements and the parental origin of most rare alleles. Clearly, the MVR and PCR length methods are complementary and when used together, provide optimal detection of structurally-altered alleles. In fact, the combination of methods uncovered an additional 8 rare alleles that would have gone undetected by length analyses alone.

The normal frequency distribution of H-ras alleles in African-American controls differed from that of white controls, despite the relatively small number of African-American subjects in this study. Such racial variation in H-ras alleles has been noted in previous studies (11,18). Weston et al (11) found the a3.5 allele to be significantly associated with the African-American population; in our study, the a3.5 allele appears to be an a4-type allele of 85 repeats. We have identified two additional alleles in African-American subjects, a1-2 (28 repeats) and a1+6 (36 repeats); these alleles occur almost as frequently as a1 and they each possess a unique MVR sequence indicating they should probably be considered common alleles.

Overall, rare alleles were not associated with breast cancer in whites, although the subset of rare alleles having lengths of 59 repeats (some of which had the A59-type sequence) appeared

to moderately increase breast cancer risk (OR=5.3, 95% CI=0.5-67.7). This increase was not statistically significant due to the small numbers of these rare alleles. Further evaluation of the 59-repeat rare alleles in a larger population of white breast cancer cases and controls is warranted. In contrast to the white population, breast cancer was significantly associated with rare H-ras alleles in African-American women (OR=5.6, 95% CI=1.2-25.8). A stronger association of H-ras rare alleles with breast cancer in African-American subjects than in white subjects was also observed in the original Southern blotting study (15).

The exact basis for the breast cancer risk heterogeneity associated with rare H-ras alleles among blacks and whites is unclear. One possibility may be a selection bias related to subject recruitment, however, this seems unlikely since black and white subjects were derived from the same clinics, and the cases and controls within each group were of similar age. Another possibility may be that the greater breast cancer risk associated with rare alleles in African Americans is due to the strategy we used to classify alleles as rare versus common. Rare length alleles were identified as those occurring at individual frequencies of $\leq$0.5% in the predominantly white combined control population, so that some low frequency alleles in the small number of African-American subjects may have been diluted and thus erroneously classified as rare. Because of the obvious allelic variation between the black and white populations, the ideal approach for H-ras allelotyping is to evaluate each group separately in order to clearly define their spectra of normal "common" alleles. This approach would reduce the bias resulting from using the white subject group as the standard. We are further exploring the breast cancer risk associated with rare alleles in African-Americans, by performing H-ras allelotyping in a much larger population-based, case-control study of breast cancer in which half the subjects are African-American.

The mechanism underlying the association of certain H-ras VNTR alleles with the development of cancer is unclear. It has been suggested that rare H-ras alleles may be in linkage disequilibrium with another gene that is responsible for the cancer risk, or they may be markers of genomic instability which predispose to cancer. In support of this latter hypothesis, an

increased frequency of microsatellite alterations was found in lung tumors from individuals with rare H-ras alleles, compared to those carrying only common alleles (19). Alternatively, the H-ras VNTR may possess a biologic function and thus directly participate in breast carcinogenesis by acting as a modifier region which affects the penetrance or expression of nearby genes (12). Interestingly, rare H-ras alleles appear to increase the penetrance of ovarian cancer in BRCA1 mutation carriers (20).

Several studies suggest that transcriptional regulatory proteins of the NF-κB/rel family bind the H-ras VNTR (21), and that this region exerts allele-specific, cell type-specific, and promoter-specific effects on reporter gene transcription (22-24). The putative regulatory effects of the H-ras VNTR may be similar to those recently described for the insulin VNTR which is located 600 bases upstream of the insulin gene. The insulin VNTR has been identified as one of several susceptibility loci (IDDM2) for type 1 insulin-dependent-diabetes (25). In Caucasians, the short, high-risk class I alleles (26 to 63 repeats) generally predispose to IDDM2 while the longer class III alleles (140 to >200 repeats) have a dominant protective effect. However, this pattern of disease susceptibility at IDDM2 is complicated by possible heterogeneity within the two allele classes as well as parent-of-origin effects (26). The insulin VNTR binds the transcription factor Pur-1 (27), and the class III alleles are associated with higher transcriptional levels of pancreatic and thymic insulin (27-29). The protective effect of the class III alleles has been explained by higher levels of insulin mRNA in thymus, leading to elevated levels of preproinsulin and enhanced immune tolerance to this protein, which is a key autoantigen in the pathogenesis of type 1 diabetes (26,28,29).

Our studies of the H-ras VNTR suggest that the spectrum of H-ras alleles varies among African-Americans and whites, and that rare alleles are associated with breast cancer primarily in African-Americans. In addition, certain rare alleles might confer a higher breast cancer risk than others, although this remains to be verified. If individual H-ras alleles vary in biological function, as has been documented with the insulin VNTR, then population variation in H-ras allele spectrum could at least partially account for their differences in breast cancer

susceptibility. Identification of a well-defined function for the H-ras VNTR and characterization of individual H-ras alleles would significantly enhance our understanding of the mechanism of association of rare alleles with cancer.

ACKNOWLEDGMENTS

This work was supported by the Breast Cancer SPORE Grant P50-CA58223 to the University of North Carolina at Chapel Hill (to K.C., B.H.S. and E.T.L.) and U.S. Army Grant DAMD17-94-J-4053 (to K.C.).

REFERENCES

1. Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H., Goeddel, D.V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature, *302*: 33-37, 1983.
2. Krontiris, T.G., DiMarino, N.A., Colb, M., Parkinson, D.R. Unique allelic restriction fragments of the human Ha-ras locus in leukocyte and tumour DNAs of cancer patients. Nature, *313*: 369-374, 1985.
3. Lidereau, R., Escot, C., Theillet, C., Champeme, M.-H., Brunet, M., Gest, J., Callahan, R. High frequency of rare alleles of the human c-Ha-ras-1 proto-oncogene in breast cancer patients. J. Natl. Cancer Inst., *77*: 697-701, 1986.
4. Corell, B., Zoll, B. Comparison between the allelic frequency distribution of the Ha-ras1 locus in normal individuals and patients with lymphoma, breast and ovarian cancer. Hum. Genet., *79*: 255-259, 1988.
5. Barkardottir, R.B., Johannsson, O.T., Arason, A., Gudnason, V., Egilsson, V. Polymorphism of the c-Ha-ras-1 proto-oncogene in sporadic and familial breast cancer. Int. J. Cancer, *44*: 251-255, 1989.
6. Hall, J.M., Huey, B., Morrow, J., Newman, B., Lee, M., Jones, E., Carter, C., Buehring, G.C., King, M.-C. Rare HRAS alleles and susceptibility to human breast cancer. Genomics, *6*: 188-191, 1990.
7. Sheng, Z.M., Martine, G., Gabillet, M., Spielman, M., Riou, G. C-Ha-ras-1 polymorphism in breast cancer patients and a control population. Oncogene Res., *2*: 245-250, 1988.
8. Wyllie, F.S., Wynford-Thomas, V., Lemoine, N.R., Williams, G.T., Williams, E.D., Wynford-Thomas, D. Ha-ras restriction fragment length polymorphisms in colorectal cancer. Br. J. Cancer, *57*: 135-138, 1988.
9. Diedrich, U., Eckermann, O., Schmidtke, J. Rare Ha-ras and c-mos alleles in patients with intracranial tumors. Neurology, *38*: 587-589, 1988.

10. Sugimura, H., Caporaso, N.E., Hoover, R.N., Resau, J.H., Trump, B.F., Lonergan, J.A., Krontiris, T.G., Mann, D.L., Weston, A., Harris, C.C. Association of rare alleles of the Harvey ras protooncogene locus with lung cancer. Cancer Res., *50*: 1857-1862, 1990.

11. Weston, A., Vineis, P., Caporaso, N.E., Krontiris, T.G., Lonergan, J.A., Sugimura, H. Racial variation in the distribution of Ha-ras-1 alleles. Molec. Carcinogenesis, *4*: 265-268, 1991.

12. Krontiris, T.G., Devlin, B, Karp, D.D., Robert, N.J., Risch, N. An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. N. Engl. J. Med., *329*: 517-523, 1993.

13. Weston, A., Godbold, J.H. Polymorphisms of H-ras-1 and p53 in breast cancer and lung cancer: a meta-analysis. Environ. Health Perspect., *105*: 919-926, 1997.

14. Conway, K., Edmiston, S.N., Hulka, B.S., Garrett, P.A., Liu, E.T. Internal sequence variations in the Ha-ras variable number tandem repeat rare and common alleles identified by minisatellite variant repeat polymerase chain reaction. Cancer Res., *56*: 4773-4777, 1996.

15. Garrett, P.A., Hulka, B.S., Kim, Y.L., Farber, R.A. HRAS protooncogene polymorphism and breast cancer. Cancer Epidemiol. Biomarkers & Prev., *2*: 131-138, 1993.

16. Ardern, J.C., Saunders, M.J., Hyde, K., Lawson, R., Liu Yin, J.A., Lucas, G.S. Polymerase chain reaction of allele frequency and loss at the Harvey ras locus in myeloid malignancies. Leukemia, *7*: 258-262, 1993.

17. Devlin, B., Krontiris, T., Risch, N. Population genetics of the HRAS1 minisatellite locus. Am. J. Hum. Genet., *53*: 1298-1305, 1993.

18. Krontiris, T.G., DiMartino, N.A., Colb, M., Mitcheson, H.D., Parkinson, D.R. Human restriction fragment length polymorphisms and cancer risk assessment. J. Cell. Biochem., *30*: 319-329, 1986.

19. Ryberg, D., Lindstedt, B.A., Zienolddiny, S., Haugen, A. A hereditary genetic marker closely associated with microsatellite instability in lung cancer. Cancer Res., *55*: 3996-3999, 1995.

20. Phelan, C.M., Rebbeck, T.R., Weber, B.L., Devilee, P., Ruttledge, M.H., Lynch, H.T., Lenoir, G.M., Stratton, M.R., Easton, D.F., Ponder, B.A.J., Cannon-Albright, L., Larsson, C., Goldgar, D.E., Narod, S.A. Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. Nature Genet., *12*: 309-311, 1996.

21. Trepicchio, W.L., Krontiris, T.G. Members of the *rel*/NF-κB family of transcriptional regulatory proteins bind the *HRAS1* minisatellite DNA sequence. Nucl. Acids Res., *20*: 2427-2434, 1992.

22. Green, M., Krontiris, T.G. Allelic variation of reporter gene activation by the HRAS1 minisatellite. Genomics, *17*: 429-434, 1993.

23. Cohen, J.B., Walter, M.V., Levinson, A.D. A repetitive sequence element 3' of the human c-Ha-ras1 gene has enhancer activity. J. Cell Physiol. (Suppl), *5*: 75-81, 1987.

24. Spandidos, D.A., Holmes, L. Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the Ha-ras1 gene. FEBS Lett., *218*: 41-46, 1987.

25. Bennett, S.T., Lucassen, A.M., Gough, S.C.L., Powell, E.E., Undlien, D.E., Pritchard, L.E., Merriman, M.E., Kawaguchi, Y., Dronsfield, M.J., Pociot, F., Nerup, J., Bouzefri, N., Cambon-Thomsen, A., Ronningen, K.S., Barnett, A.H., Bain, S.C., Todd, J.A. Susceptibility to human type 1 diabetes at *IDDM2* is determined by tandem repeat variation at the insulin gene minisatellite locus. Nature Genet., *9*: 284-292, 1995.

26. Bennett, S.T., Wilson, A.J., Esposito, L., Bouzekri, N., Undlien, D.E., Cucca, F., Nistico, L., Bussetti, R., the IMDIAB Group, Bosi, E., Pociot, F., Nerup, J., Cambon-Thomsen, A., Pugliese, A., Shield, J.P.H., McKinney, P.A., Bain, S.C., Polychronakos, C., Todd, J.A. Insulin VNTR allele-specific effect in type 1 diabetes depends on identity of untransmitted paternal allele. Nature Genet., *17*: 350-352, 1997.

27. Kennedy, G.C., German, M.S., Rutter, W.J. The minisatellite in the diabetes susceptibility locus *IDDM2* regulates insulin transcription. Nature Genet., *9*: 293-298, 1995.

28. Vafiadis, P., Bennett, S.T., Todd, J.A., Nadeau, J., Grabs, R., Goodyer, C.G., Wickramasinghe, S., Colle, E., Polychronakos, C. Insulin expression in human thymus is modulated by *INS* VNTR at the IDDM2 locus. Nature Genet., *15:* 289-292, 1997.

29. Pugliese, A., Zeller, M., Fernandez, A., Jr., Zalcberg, L.J., Bartlett, R.J., Ricordi, C., Pietropaolo, M., Eisenbarth, G.S., Bennett, S.T., Patel, D.D. The insulin gene is transcribed in the human thymus and transcriptional levels correlate with allelic variation at the *INS* VNTR-*IDDM2* susceptibility locus for type 1 diabetes. Nature Genet., *15*: 293-297, 1997.

FIGURE LEGENDS

Figure 1:

Examples of PCR amplification of the H-ras VNTR from heterozygous samples or cloned markers. The allelotypes identified in numbers of repeats are: lane 1; 28/30, lane 2; 29/30, lane 3; 30 (single cloned marker), lane 4; 30/32, lane 5; 30/34, lane 6; 30/38, lane 7; 30/44, lane 8; 34/46, lane 9; 30/53, lane 10; 34/60, lane 11; 28/69 (mixture of two cloned markers), lane 12; 73/85, lane 13; 30/86, lane 14; 88 (single cloned marker), lane 15; 59/94. Molecular size markers are: M; Boehringer Mannheim marker VII, and m; 123 bp ladder from Gibco-BRL.

Figure 2:

Frequency distribution of H-ras allele lengths in black or white breast cancer cases and controls. Allele lengths are given in numbers of 28 bp repeats. Allele frequencies are given in percent of alleles within each group. The frequency of a1 (30 repeat) alleles for each group except the black control group is given above the a1 peak, and the number (n) of alleles within each group is given in the upper right corner of each graph. Rare alleles are indicated by *. Note that in the white control group, one rare allele having a length of 114 repeats is not shown.

Figure 3:

5' MVR analysis of the H-ras VNTR in heterozygous samples with sequence allelotypes of (A) A1aI-3/A1.2, (B) A1a/A3b, or (C) A1a/A3c. Lanes 1-4 indicate the repeat-specific primer used. Repeat 3, the first readable repeat, as well as repeat 20 are indicated by arrows.

Table 1: H-ras Allele Length Distributions in Breast Cancer Cases and Controls

Length in No. Repeats	Allele[a] Length Classi-fication	Allele Frequency					
		No. in Cases (%)			No. in Controls (%)		
		Black	White	Total	Black	White	Total*
28 (a1-2)	C	4 (11.1)	0	4 (1.3)	7 (14.6)	0	7 (1.8)
29	C	2 (5.6)	7 (2.5)	9 (2.9)	5 (10.4)	3 (0.9)	8 (2.0)
30 (a1)	C	11 (30.6)	151 (54.3)	162 (51.6)	9 (18.8)	201 (57.8)	210 (53.0)
31	C	0	7 (2.5)	7 (2.2)	1 (2.1)	9 (2.6)	10 (2.5)
32 (a1+2)	C	0	10 (3.6)	10 (3.2)	0	7 (2.0)	7 (1.8)
34 (a1+4)	C	0	4 (1.4)	4 (1.3)	1 (2.1)	5 (1.4)	6 (1.5)
36 (a1+6)	C	3 (8.3)	1 (0.4)	4 (1.3)	6 (12.5)	1 (0.3)	7 (1.8)
37	R	0	1 (0.4)	1 (0.3)	0	0	0
38	R	1 (2.8)	0	1 (0.3)	0	1 (0.3)	1 (0.3)
39	R	0	1 (0.4)	1 (0.3)	0	0	0
41	R	0	0	0	0	1 (0.3)	1 (0.3)
44	R	0	1 (0.4)	1 (0.3)	0	0	0
45	C	0	1 (0.4)	1 (0.3)	1 (2.1)	2 (0.6)	3 (0.8)
46 (a2)	C	0	34 (12.2)	34 (10.8)	0	38 (10.9)	38 (9.6)
47	C	1 (2.8)	3 (1.1)	4 (1.3)	0	5 (1.4)	5 (1.3)
55	R	1 (2.8)	1 (0.4)	2 (0.6)	0	0	0
59	R	3 (8.3)	4 (1.4)	7 (2.2)	1 (2.1)	1 (0.3)	2 (0.5)
61	R	1 (2.8)	0	1 (0.3)	0	0	0
66	R	3 (8.3)	0	3 (1.0)	1 (2.1)	0	1 (0.3)
67	R	0	0	0	0	1 (0.3)	1 (0.3)
68	R	0	0	0	0	1 (0.3)	1 (0.3)
69 (a3)	C	0	23 (8.3)	23 (7.3)	0	27 (7.8)	27 (6.8)
70	C	0	5 (1.8)	5 (1.6)	1 (2.1)	4 (1.1)	5 (1.3)
72	R	0	0	0	0	1 (0.3)	1 (0.3)
73	R	0	0	0	0	1 (0.3)	1 (0.3)
78	R	0	0	0	0	1 (0.3)	1 (0.3)
80	R	1 (2.8)	0	1 (0.3)	0	0	0
81	R	0	1 (0.4)	1 (0.3)	0	0	0
85 (a4)	C	2 (5.6)	2 (0.7)	4 (1.3)	6 (12.5)	1 (0.3)	7 (1.8)
86 (a4)	C	0	8 (2.9)	8 (2.5)	0	4 (1.1)	4 (1.0)
87 (a4)	C	0	11 (4.0)	11 (3.5)	0	23 (6.6)	23 (5.8)
88 (a4)	C	2 (5.6)	2 (0.7)	4 (1.3)	8 (16.7)	9 (2.6)	17 (4.3)
89	R	0	0	0	1 (2.1)	0	1 (0.3)
94	R	1 (2.8)	0	1 (0.3)	0	0	0
114	R	0	0	0	0	1 (0.3)	1 (0.3)
Total		36	278	314	48	348	396

[a]Ha-ras alleles are classified by PCR length analysis as common (C), having individual frequencies >0.5%, or rare (R), having individual frequencies of ≤0.5% in the total control group*.

Table 2: H-ras 5' MVR Sequence Distributions in Breast Cancer Cases and Controls

5' MVR Pattern	5' MVR Allele Sequence: MVR Repeat Sequence (repeats 1-20)	Number of Alleles: Case	Control	Total	Allele Lengths Associated with Each MVR Pattern: Length in No. Repeats (No. Alleles)
Common MVR Patterns					
A1.02 (a1-2)	5'-001321.11311313121131	4	7	11	28 (11)
A1a (a1)	5'-001321.13113221311222	138	187	325	30 (310), 29 (11), 31 (1), 32 (1), 34 (1), 39 (1)
A1b (a1)	5'-001321.13113221312122	31	37	68	30 (59), 31 (8), 29 (1)
A1.2 (a1+2)	5'-001321.13113221223112	9	5	14	32 (14)
A1.4 (a1+4)	5'-001321.13113221324131	4	4	8	34 (8)
A1.6 (a1+6)	5'-001321.22212113113221	3	6	9	36 (9)
A2 (a2)	5'-001321.22221311313123	37	44	81	46 (68), 47 (9), 45 (2), 55 (1), 72 (1)
A3a (a3)	5'-001321.21132141224123	10	11	21	69 (10), 70 (6), 66 (4), 59 (1)
A3b (a3)	5'-001321.21132111241231	13	11	24	69 (21), 30 (1), 59 (1), 68 (1)
A3c (a3)	5'-001321.21132141324123	5	7	12	69 (9), 70 (3)
A4 (a4)	5'-001321.41224123241222	26	49	75	87 (34), 88 (17), 85 (11),86 (10), 89 (1), 94 (1), 114 (1)
Variant MVR Patterns					
A1aC-3	5'-001321.13113221312222	0	1	1	30 (1)
A1aD-1	5'-001321.13112213112223	1	0	1	29 (1)
A1aD-2	5'-001321.13113221311223	1	1	2	29 (2)
A1aI-1	5'-001321.12311322131122	0	1	1	31 (1)
A1aI-2	5'-001321.13113222131122	1	0	1	31 (1)
A1aI-3	5'-001321.13113221312112	0	1	1	32 (1)
A1aI-4	5'-001321.11311322131122	0	1	1	31 (1)
A1bC-1	5'-001321.11113221312122	1	0	1	46 (1)
A1bC-4	5'-001321.43113221312122	0	1	1	30 (1)
A1bD-1	5'-001321.13113221121222	1	0	1	29 (1)
A1bD-2	5'-001321.11132213121222	1	0	1	29 (1)
A1bI-1	5'-001321.13311322131212	1	1	2	31 (2)
A1bI-3	5'-001321.11311322131212	3	0	3	31 (3)
A1.2C-1	5'-001321.13113221223132	1	0	1	36 (1)
A1.4C-1	5'-001321.13113221314131	0	1	1	34 (1)
A2C-1	5'-001321.02221311313123	1	1	2	46 (2)
A2C-3	5'-001321.12221311313123	1	1	2	44 (1), 46 (1)
A2D-2	5'-001321.22221311313131	1	1	2	45 (2)
A3d	5'-001321.21132113241231	2	2	4	69 (3) ,73 (1)
A3aC-1	5'-001321.21232141224123	1	0	1	69 (1)
A3aD-1	5'-001321.21132112241231	2	1	3	69 (3)
A3aM-1	5'-00131.131133213112223	0	2	2	69 (2)
A3bC-3	5'-001321.21132110241231	1	0	1	70 (1)
A3bC-4	5'-001321.21132111241234	0	1	1	69 (1)
A3dD-1	5'-0011.21132141124123l4	0	1	1	67 (1)
A4C-2	5'-001321.41224123241221	3	4	7	86 (2), 88 (4), 80 (1)
Rare MVR Patterns					
A1R-1	5'-001321.13113221311322	1	0	1	37 (1)
A1R-2	5'-001321.13113221223131	0	1	1	36 (1)
A1.8	5'-001321.22221131231413	1	1	2	38 (2)
A59-1	5'-001321.23113214232412	1	1	2	59 (2)
A59-2	5'-001320.23113214123241	1	0	1	59 (1)
A59-3	5'-001321.23113214233412	1	0	1	59 (1)
A3R-1	5'-001321.21132121132121	1	0	1	81 (1)
A4R-1	5'-001321.41224123133241	4	1	5	59 (3), 55 (1), 61 (1)
A4R-3	5'-001321.41222221312433	0	1	1	78 (1)
U-1	5'-001321.11113311311311	0	1	1	41 (1)
U-2	5'-001321.41231132213112	1	0	1	32 (1)
Total Alleles		314	396	710	

MVR patterns are classified as common, variant, or rare. Each common MVR pattern is strictly associated with a specific allele length. The variant or rare pattern names incorporate D, I, C, M, R or U to indicate the sequence alteration relative to the common progenitor allele, if known. Variant MVR patterns (D; deletion, I; insertion, C; change of repeat type, M; multiple (two) changes) have one or two repeat differences out of 20 repeats compared with the common progenitor allele. Rare alleles deviate by more than two repeats compared with the common progenitor allele. The U-1 and U-2 rare alleles are unique in sequence and appear unrelated to other common or rare alleles.

Table 3: Structural Characterization of the Common and Rare H-ras Alleles

Common Allele or Subject Number	Allele Length (repeats)	*5' MVR Pattern Indicating Allelic Origin	**5' MVR Sequence (repeats 1-20)	Relationship Between Allele Length (L) and MVR Sequence (S)				
				Common Alleles	Rare Alleles			
				Matched CL+CS	RL+RS	RL+CS	CL+RS	Mismatched CL+CS
Common Alleles* **								
a1-2	28	A1.02	5'-001321.11311313121131	+	--	--	--	--
a1	30	A1a	5'-001321.13113221311222	+	--	--	--	--
a1	30	A1b	5'-001321.13113221312122	+	--	--	--	--
a1+2	32	A1.2	5'-001321.13113221223112	+	--	--	--	--
a1+4	34	A1.4	5'-001321.13113221324131	+	--	--	--	--
a1+6	36	A1.6	5'-001321.22212113113221	+	--	--	--	--
a2	46	A2	5'-001321.22221311313123	+	--	--	--	--
a3	69	A3a	5'-001321.21132141224123	+	--	--	--	--
a3	69	A3b	5'-001321.21132111241231	+	--	--	--	--
a3	69	A3c	5'-001321.21132141324123	+	--	--	--	--
a4	85-88	A4	5'-001321.41224123241222	+	--	--	--	--
Rare Alleles								
Black Case								
415	38	A1.8 (A2?)	5'-001321.22221**131231413**	--	+	--	--	--
--	55	A4R-1	5'-001321.41224123**133241**	--	+	--	--	--
166	59	A4R-1	5'-001321.41224123**133241**	--	+	--	--	--
421	59	A4R-1	5'-001321.41224123**133241**	--	+	--	--	--
--	94	A4	5'-001321.41224123241222	--	--	+	--	--
425	59	A59-2	5'-00132**0.23113214123412**	--	+	--	--	--
--	61	A4R-1	5'-001321.41224123**133241**	--	+	--	--	--
335	66	A3a	5'-001321.21132141224123	--	--	+	--	--
527	66	A3a	5'-001321.21132141224123	--	--	+	--	--
500	66	A3a	5'-001321.21132141224123	--	--	+	--	--
565	80	A4C-2	5'-001321.41224123241221	--	--	+	--	--
Black Control								
45	59	A4R-1	5'-001321.41224123**133241**	--	+	--	--	--
604	66	A4C-2	5'-001321.41224123241221	--	--	+	--	--
419	89	A4	5'-001321.41224123241222	--	--	+	--	--
White Case								
544	30	A3b	5'-001321.21132111241231	--	--	--	--	+
462	32	U-2 (A4?)	5'-001321.412**31132213112**	--	--	--	+	--
302	36	A1.2C-1	5'-001321.13113221223132	--	--	--	--	+
136	37	A1R-1	5'-001321.131132213113**22**	--	+	--	--	--
51	39	A1a	5'-001321.13113221311222	--	--	+	--	--
402	44	A2C-3	5'-001321.12221311313123	--	--	+	--	--
59	46	A1bC-1	5'-001321.11113221312122	--	--	--	--	+
116	55	A2	5'-001321.22221311313123	--	--	+	--	--
542	59	A3b	5'-001321.21132111241231	--	--	+	--	--
529	59	A59-3	5'-001321.**23113214233412**	--	+	--	--	--
379	59	A59-1	5'-001321.**23113214232412**	--	+	--	--	--
194	59	A3a	5'-001321.21132141224123	--	--	+	--	--
348	81	A3R-1	5'-001321.211321**21132121**	--	+	--	--	--
White Control								
97	32	A1a	5'-001321.13113221311222	--	--	--	--	+
490	32	A1aI-3	5'-001321.13113221312112	--	--	--	--	+
238	34	A1a	5'-001321.13113221311222	--	--	--	--	+
343	36	A1R-2	5'-001321.131132212**23131**	--	--	--	+	--
512	38	A1.8 (A2?)	5'-001321.22221**131231413**	--	+	--	--	--
37	41	U-1 (?)	5'-001321.**11113311311311**	--	+	--	--	--
287	59	A59-1	5'-001321.**23113214232412**	--	+	--	--	--
513	67	A3dD-1	5'-001121.13214112412314	--	--	+	--	--
39	68	A3b	5'-001321.21132111241231	--	--	+	--	--
499	72	A2	5'-001321.22221311313123	--	--	+	--	--
458	73	A3d	5'-001321.21132113241231	--	--	+	--	--
397	78	A4R-3	5'-001321.4122**2221312433**	--	+	--	--	--
292	114	A4	5'-001321.41224123241222	--	--	+	--	--

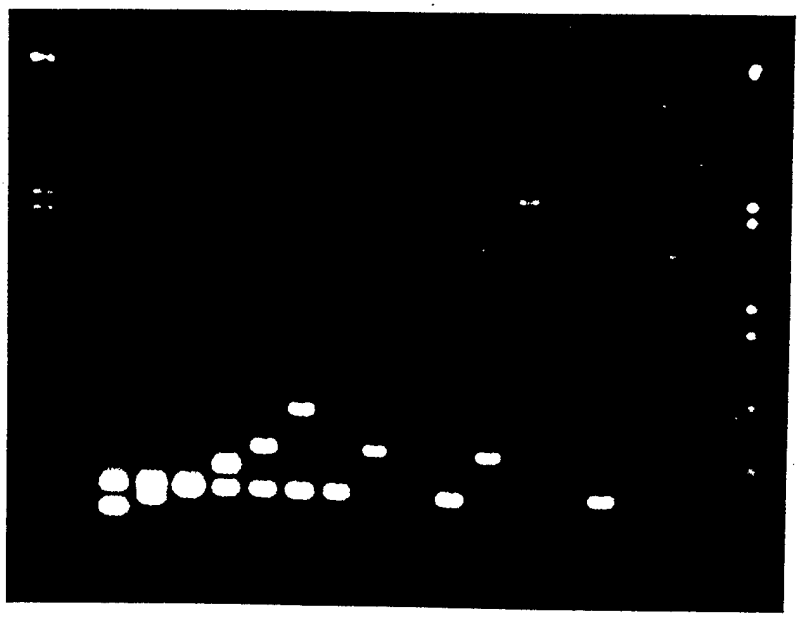
*The underlined portion of the MVR pattern indicates the common progenitor allele. If this cannot be definitely established, the suspected progenitor allele is given in parentheses. **The underlined sequence indicates that portion of the rare allele which varies from the common progenitor sequence. ***Common alleles with minor sequence variations are considered CL+CS (not shown); see Table 2 for a listing of all MVR variants. CL; common length, CS; common MVR sequence, RL; rare length, RS; rare MVR sequence.

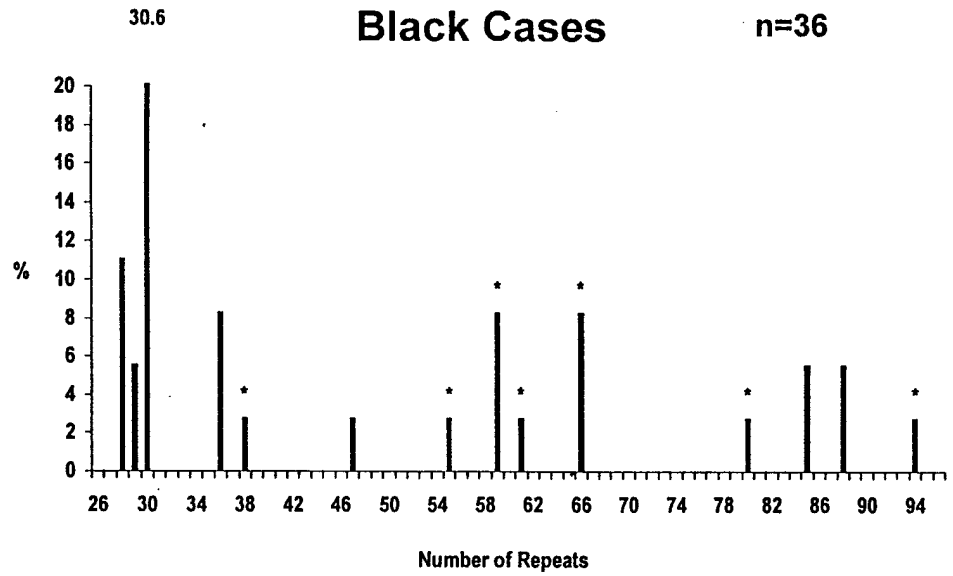
Table 4: The Effect of Rare H-ras Alleles Identified by PCR-Based Methods on Breast Cancer Risk

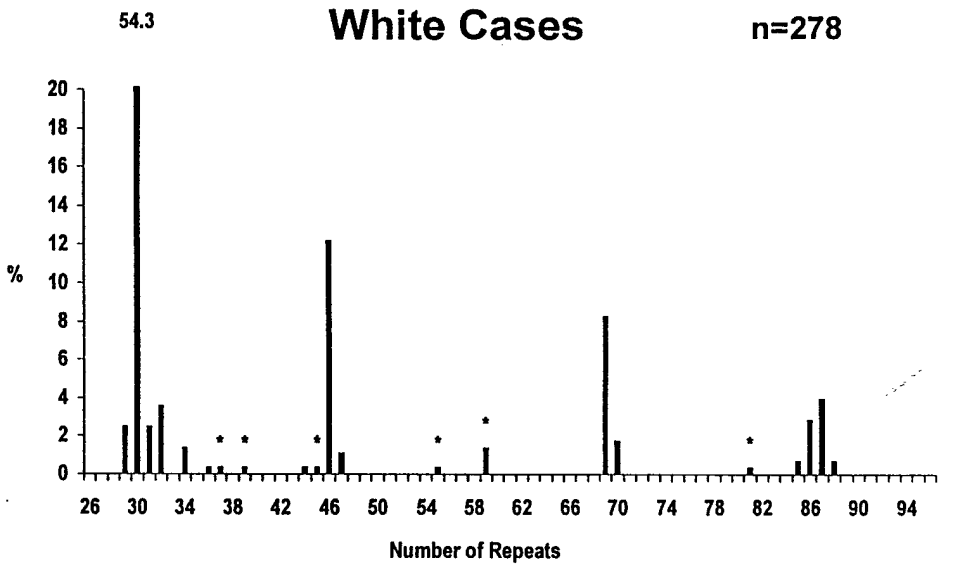
Subjects	Rare by Length Only[a]		Rare by MVR Only[b]		Rare by Both Length and MVR[c]		Rare by Any Criteria[d]		Total Subjects
	Rare No. (%)	Common No. (%)	Rare No. (%)	Common No. (%)	Rare No. (%)	Common No. (%)	Rare No. (%)	Common No. (%)	
Black									
Case	*8 (44.4)	10 (55.6)	*4 (22.2)	14 (77.8)	*4 (22.2)	14 (77.8)	*8 (44.4)	10 (55.6)	18
Control	3 (12.5)	21 (87.5)	1 (4.2)	23 (95.8)	1 (4.2)	23 (95.8)	3 (12.5)	21 (87.5)	24
Total	11 (26.2)	31 (73.8)	5 (11.9)	37 (88.1)	5 (11.9)	37 (88.1)	11 (26.2)	31 (73.8)	42
OR	5.6		6.6		6.6		5.6		
95% CI	1.2-25.8		0.7-64.9		0.7-64.9		1.2-25.8		
White									
Case	9 (6.5)	130 (93.5)	5 (3.6)	134 (96.4)	4 (2.9)	135 (97.1)	13 (9.4)	126 (90.6)	139
Control	9 (5.2)	165 (94.8)	5 (2.9)	169 (97.1)	6 (3.4)	168 (96.6)	13 (7.5)	161 (92.5)	174
Total	18 (5.8)	295 (94.2)	9 (2.9)	304 (97.1)	10 (3.2)	303 (96.8)	26 (8.3)	287 (91.7)	313
OR	1.3		1.3		0.8		1.3		
95% CI	0.5-3.3		0.3-4.5		0.2-3.0		0.6-2.9		
Combined									
Case	*17 (10.8)	140 (89.2)	*9 (5.7)	148 (94.3)	*8 (5.1)	149 (94.9)	*21 (13.4)	136 (86.6)	157
Control	12 (6.1)	186 (93.9)	6 (3.0)	192 (97.0)	7 (3.5)	191 (96.5)	16 (8.1)	182 (91.9)	198
Total	29 (8.2)	326 (91.8)	14 (3.9)	341 (96.1)	15 (4.8)	338 (95.2)	37 (10.4)	318 (89.6)	355
OR	1.9		2.0		1.5		1.8		
95% CI	0.9-4.1		0.7-5.6		0.5-4.1		0.9-3.5		

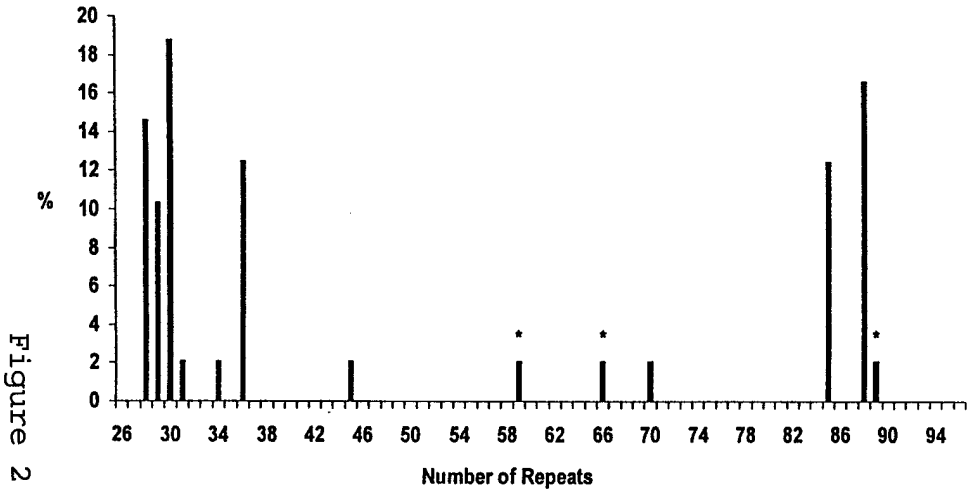
Risk analyses were performed by subject. [a]Rare alleles using length criteria only (RL+CS and RL+RS),have individual frequencies of ≤0.5%, and were identified in Table 1. [b]Rare alleles using MVR sequence criteria only (CL+RS and RL+RS) differed from the common progenitor allele by more than two repeats out of 20 and were identified in Table 3 . The minor MVR variants, having 1 or 2 repeat differences, were considered common for these analyses. [c]Rare alleles having both rare length and rare sequence (RL+RS). [d]All rare alleles by any criteria; RL+CS, CL+RS, RL+RS CL+RS and mismatched CL+CS. *Note that three subjects possessed two rare alleles. OR; odds ratio. CI; confidence interval.

Figure 1




30.6
Black Cases
n=36
%
Number of Repeats


54.3
White Cases
n=278
%
Number of Repeats


Black Controls
n=48
%
Number of Repeats

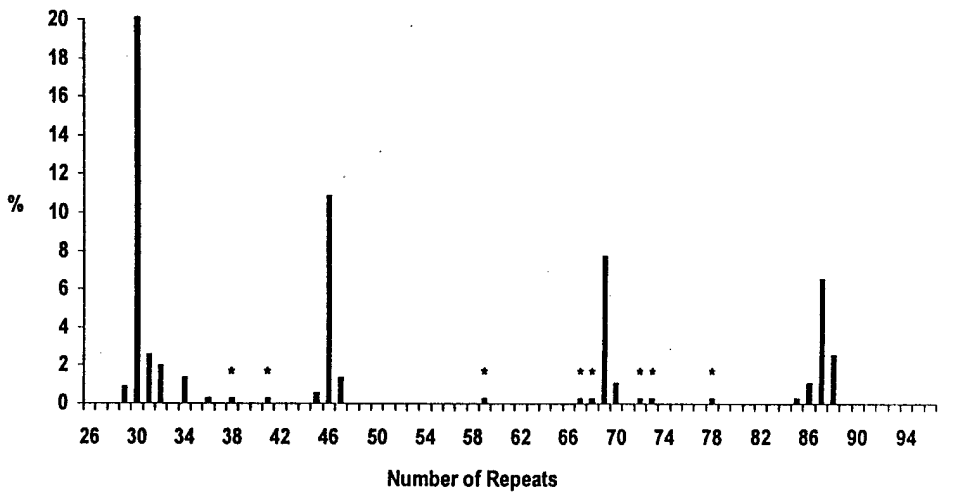

57.8
White Controls
n=348
%
Number of Repeats

Figure 2

Figure 3