Quantitation of early clonal expansion of two mutant 61st codon c-Ha-ras alleles in DMBA/TPA treated mouse skin by nested PCR/RFLP

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It has been hypothesized that tumor promotion in mouse skin involves clonal expansion of initiated cells with activated c-Harvey (Ha)-ras oncogene to give rise to benign tumors. We have used the two stage mouse skin carcinogenesis model using 7,12-dimethylbenz[a]anthracene (DMBA) as the initiator and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as the tumor promoter to quantitate the number of mutated c-Ha-ras alleles in mouse epidermal DNA. Epidermal samples were harvested over a 12-week period before the appearance of papillomas. Three 61st codon (i.e. CAA) c-Ha-ras mutations, CTA (T2), CGA (G2) and CAT (T3) were quantitated by newly developed nested PCR/RFLP assays. During TPA promotion the number of T2 mutant copies showed a progressive increase starting at 4 weeks after initiation and the number of T3 mutant alleles showed an increase starting at 6 weeks. By 12 weeks after initiation, TPA-promoted mouse epidermis averaged ~8 × 10^4 T2 mutant alleles per epidermis while the number of T3 mutant alleles averaged 3 × 10^4 per epidermis. The best-fit lines for the quantitation of mutant alleles derived from DMBA/TPA-treated mice from 4 to 12 weeks after initiation were exponential. These results were consistent with clonal expansion of epidermal cells carrying these mutations during tumor promotion. The slopes of the best-fit lines for the mutant copies indicated a trend in which cells with the T2 mutations had a growth advantage during TPA promotion over cells with the T3 mutation.

Introduction

Mouse skin carcinogenesis has been divided into three stages as an operational basis of understanding the multiple cellular and molecular alterations involved. The first stage, initiation, appears to involve mutational events. The second stage, promotion, involves epigenetic changes that contribute to the clonal expansion of the initiated cells. Tumor progression involves further genetic changes that allow for acquisition of malignant characteristics such as invasion, metastasis, angiogenesis and genetic instability (1).

Point mutations in the ras family of proto-oncogenes have been observed in human tumors (2) as well as in experimentally induced animal tumors (3). The two-step model of mouse skin carcinogenesis uses 7,12-dimethylbenz[a]anthracene (DMBA*) as an initiator, followed by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as a promoter (1). This established model continues to be used to determine the molecular and cellular mechanisms involved in chemical and radiation carcinogenesis and to develop methodology for less defined situations. The first step in this model, the formation of DMBA-DNA adducts, results in characteristic mutations (4). Mutations in the 61st codon of the c-Ha-ras gene are the most frequent mutations documented with this chemical initiating agent (5,6). An accepted hypothesis is that the activating ras mutations cause alterations in the ras pathway that allow the clonal expansion of mutant cells under the influence of TPA (7). TPA has been shown to induce a mitogenic response in some basal keratinocytes while inducing terminal differentiation in others (8). It has been proposed that the activating mutations cause cells to remain in a proliferating state and resist terminal differentiation (9). Co-culture of cells with activated 61st codon c-Ha-ras and normal keratinocytes shows a dose dependent outgrowth of colonies with TPA promotion (10).

Three different 61st codon mutations have been noted in papillomas that eventually appear using the DMBA/TPA protocol. The majority (94%) of the papillomas have an A to T mutation in the 2nd position of 61st codon (T2) (11). Rarely found are A to G mutations in the second position of the 61st codon (G2) (4%) and the A to T mutations in the 3rd position (T3) (2%) (6).

An increased frequency of the T2 mutations soon after DMBA initiation, which by differential adduct formation or selective repair, is a possible explanation for the preponderance of papillomas with this mutation. Another possible explanation is a growth advantage for cells with the T2 mutation over the other mutations during promotion with TPA. Quantitating the specific c-Ha-ras mutations during early phases of tumor promotion might differentiate between these two possibilities. Although 61st codon mutations have been detected prior to papilloma development, no determination of the relative frequency of these three specific mutations has been made early in tumorigenesis. In this work we describe quantitation of these specific 61st codon c-Ha-ras mutations in mouse epidermis after initiation with DMBA and during the early stages of TPA promotion prior to the development of visible benign papillomas.

Materials and methods

Treatment of animals

The two-step mouse skin model of DMBA initiation followed by TPA promotion was used. For the dose-response/time dependence data, 5-week-old female CD-1 mice (Charles River) were topically treated on shaved dorsal skin with 5 μg (20 nmol), 30 μg (200 nmol) and 200 μg (800 nmol) of the initiator DMBA (Sigma) in 200 μl of acetone followed by TPA promotion (Chemicals for Cancer Research, Chanhassen, MN) at 10 μg (17 nmol) once per week starting 1 week after DMBA (12). Two mouse skins were harvested and the epidermis was separated from dermis by heat shock (13) before being pooled into one sample. In a second study, the dose of 50 μg of DMBA (14) was used to make replicate samples for each treatment group and harvest time. Four groups were studied including untreated animals, TPA promotion only, DMBA initiation only, and DMBA plus TPA. Samples were taken at various times for up to 12 weeks.
DNA purification and fragmentation

DNA was purified by proteinase K and RNase treatment, followed by phenol/chloroform/isoamyl alcohol extractions. High molecular weight DNA was precipitated, dissolved in water, and quantitated by spectrophotometry. DNA was digested at 37°C for 3 h with 5 units PstI/φX174 DNA and 5 units HindIII/φX174 DNA at a concentration of 0.2 μg DNA/μl.

Primers

Based on published sequence (16), first stage primers were chosen to amplify the same region of exon 2 of mouse c-Ha-ras for the detection of all three mutations.

Stage 1 Upstream Primer-5’ CGTTGAGTTCTGGTCTGAGGGG3’ (bp 244–267).
Stage 1 Downstream Primer-5’ TGAGTGGCTACCTGACTG 3’ (bp 491–510).

Second stage primers were different for the different assays. For both 61st codon mutation assays, 2nd position A to T (T2) and second position A to G (G2).

Stage 2 Upstream Primer-5’ CGAGCCCTTGGTTTGGACAGGC3’ (bp 302–322).
Stage 2 Downstream Primer-5’ GGAG GCCTTTGTTGATGGGC3’ (bp 455–475).

For detection of the third position A to T (T3) mutations, primers were designed to remove NlaIII sites found in both mutant and wild-type alleles and then proceeded to produce a longer mutation specific NlaIII-digestion product (mismatches underlined).

Stage 2 Upstream Primer-5’ TGTCTCTGTGAGGAACCGT (bp 739–760).
Stage 2 Downstream Primer-5’ TGACTGTGCCCGCGTG (bp 805–822).

Nested PCR/RFLP protocol

The nested PCR/RFLP protocol (17) produces a radio-labeled PCR product of 267 bp in the first stage of the assays for the three mutations. A single radiolabeled band of 176 bp is made in the second stage of the T2 and G2 assays. When double stranded products from the first extensions are digested with XhoI (for T2) or TaqI (for G2), only the products from the template with mutant 61st codons are digested. Digested products form a doublet of 91 and 85 bp for T2 and 90 and 86 bp for G2. In the T3 assay, the second stage double stranded PCR product of 84 bp forms specific bands of 52 and 32 bp after NlaIII digestion (Figure 1).

The first stage of PCR includes 500 ng of DNA with 2 mM MgCl2 from the digestion buffer, 0.3 μM outside primers, 1 X UThma Buffer (Perkin Elmer) and 4 μM each of dNTP in a reaction volume of 50 μl.

The reaction was started with the addition of 2.4 units of UThma polymerase (Perkin Elmer) into preheated DNA mix. Initial denaturation was for 2 min at 97°C, followed by 20 cycles of 1 min at 97°C, 1 min at 60°C and 1 min at 72°C. Auto-32
extension of 30 s/cycle after 10 cycles, and final extension of 20 min at 72°C. The second stage of PCR included 10 μl of a 1:100 dilution of the first stage product is digested with NlaIII to produce diagnostic fragments of 52 and 32 bp after NlaIII digestion (Figure 1).

Diagnostic restriction fragmentation and separation of the fragments

The second stage product (10 μl) was added with or without 10 units of mutation specific enzyme (XhoI, TaqI or NlaIII) into a total volume of 30 μl digestion buffer and incubated 3–5 h under oil. Aliquots of 8 μl of digestion buffer were added and 10 μl loaded onto a 12% gel, 0.4 mm thick 1X TBE/1 X UThma Buffer (American Technologies). Electrophoresis was done for 6 h at 400 V. Gels were dried for 30 min at 80°C.

Phosphor imaging and processing of data

The gel was exposed to a phosphor imaging plate (Molecular Dynamics) for 48–64 h for the mutation specific band and 1–3 h for the undigested portion of the PCR product. Diagnostic product was quantitated with ImageQuan® (Molecular Dynamics) software using a background subtraction from each lane, and normalized to the undigested PCR product. Comparison to the standard curve yielded a number representing the copies of mutant alleles in a sample of 500 ng of DNA. Total DNA recovered from an epidermis, 400 μg in mice receiving TPA treatment, was used as a factor to convert mutant copies/500 ng to mutant copies/epidemi.
nested PCR/RFLP assays were developed as shown in Figure 1. A phosphor imaged D-600 polyvinyl gel (Figure 2) shows the characteristics of dilutions of these plasmid standards (T2, G2 and T3). Weak diagnostic radioactive bands were observed with untreated epidermal DNA for all three mutations before UITma® was introduced into the protocol. Although they were greatly reduced in the assays using UITma® and low nucleotide concentration, they remained a problem particularly in the G2 assay. Further testing showed these bands detected in the G2 assay were formed during the assay. Digestion of the genomic DNA with the mutation-specific enzyme TaqI before the assay destroyed pre-existing mutations, but no reduction of this band using DNA from untreated animals could be seen with digestion (data not shown).

Quantitation of the dilutions of mutant plasmids from 500 copies per assay to 15 copies (1 mutant copy per 10⁴ wild-type copies) is shown in Figure 3 for all three assays. Fresh dilutions of these standards were processed each time along with a set of biological samples. The T2 assay (Figure 2) had the least background with the control sample for the 85 bp band and this band was therefore used to quantitate this mutation. The slope of the line changed below 15 copies (Figure 2, Figure 3A). All values for mutant copy numbers were determined by reading directly off a line much like the line shown in Figure 3A. Using the lower 86 bp band in the G2 assay (Figure 2, Figure 3B), the 15 copy sample (1 mutant per 10⁴ wild type) was not reliably higher than control. The T3 mutation assay had the disadvantage that the diagnostic enzyme NlaIII cut the PCR band into four small pieces. Therefore primers were included in the second stage of this assay to remove two NlaIII sites and leave only the mutation specific site (Figure 1). For each mutation assay, no cross-reactivity occurred with 500 copies of the other mutant plasmids (Figure 2).

Time-course study for T2 mutations in the epidermis of mice initiated with different doses of DMBA and promoted with TPA

Before a DMBA dose was chosen for performing a more extensive biological study of DMBA initiation and TPA promotion, a pilot experiment at three dose levels of DMBA was performed. Two subcarcinogenic dose levels (14), 5 µg (20 nmol) and 50 µg (200 nmol), and a complete carcinogenic dose of 200 µg (800 nmol) were used. Samples were taken at 1 week post-initiation and after promotion with TPA at 4 weeks, 8 weeks, and 12 weeks. The skin appeared hyperplastic at 12 weeks but there were no papillomas visible. A rise in detected mutant c-Ha-ras copies represents an increase in the proportion of cells bearing the mutation of interest. Figure 4 shows the dose-response of the 61st codon second position A to T mutation (T2) in the epidermis taken at 8 and 12 weeks (after 7 and 11 weeks of weekly TPA promotion). This dose response was also seen at 4 weeks (data not shown). The higher subcarcinogenic dose of 50 µg (200 nmol) of DMBA was chosen to study the effects of TPA promotion on expansion of the three mutant c-Ha-ras mutant copies in the following experiments.

Time-course for the CTA (T2) 61st codon mutation of c-Ha-ras after DMBA initiation and TPA promotion

Three independent sets of epidermal DNAs that included all treatment groups and times were processed along with standards. The assays were run on gels at the same time to allow for day to day, or gel to gel variations. Figure 5 shows the data for this mutation as a scatter plot to visually demonstrate the variability between the samples. Samples that showed no mutant copies have been displaced slightly on both axes to show all the samples. Samples of epidermal DNA, derived from mice that were both initiated with 50 µg (200 nmol) DMBA and promoted with TPA, showed an outgrowth of the A to T mutation in the 2nd position of the 61st codon of c-Ha-ras (T2). All data points for the untreated animals, TPA only, and DMBA only are shown to demonstrate the
background of the assay. The mutant copies reached a level of \(-8 \times 10^6\) mutant cells per epidermis in the sample by 12 weeks with this DMBA/TPA regimen. The best-fit line using least square analysis for the T2 mutation is expressed by an equation in the form \(y = \exp (i + gt)\) where \(y = \text{mutant copies; } i = \text{mutations at initiation}, g = \text{growth rate under TPA conditions and } t = \text{time in weeks is } y = \exp (0.305 + 0.552 i). This simple equation for exponential growth would be useful only in the early stages of carcinogenesis during the growth of mutant cells in a background of wild-type cells. Limitations because of the arrangement of cells or other local effects are assumed to be minimal in this protocol before the formation of papillomas. The correlation coefficient, \(r\), for fit of the line is 0.789.

**Time-course for the CGA (G2) 61st codon mutation of\( c\)-Ha-ras after DMBA initiation and TPA promotion**

Figure 6 shows the data for the G2 mutation plotted in the same manner as the T2 data. No consistent increase in G2 mutations could be seen with time. In addition, no consistent difference in G2 mutation levels was observed between treatment groups over the course of the 12 week experiment.

**Time-course for the CAT (T3) 61st codon mutation of\( c\)-Ha-ras after DMBA initiation and TPA promotion**

Figure 7 shows the data for the T3 mutation plotted in the same manner as the graphs for the other two mutations. Some of the DMBA plus TPA samples showed mutant copies above the background by 5 and 6 weeks with outgrowth clear in the group at 12 weeks. At 12 weeks an average of \(\sim 40\) copies, \(i = \text{mutations at initiation}, g = \text{growth rate under TPA conditions and } t = \text{time in weeks for the T3 mutation is } y = \exp (0.301 + 0.276 i). The correlation coefficient, \(r\), for the fit of the line is 0.875.
of the normal signaling in the ras pathway, increased prolifera-
tion of cells with mutant c-Ha-ras alleles and/or increased
terminal differentiation of normal cells.

With this DMBA/TPA protocol, we quantitated ~8x10^5 T2
mutant alleles/epidermis at 12 weeks and only 3x10^6 T3
mutant alleles/epidermis at the same time. This is consistent
with the reported preponderance of the T2 papillomas (94%)
and the low number of T3 papillomas (2%) eventually observed
(6). Two explanations can be proposed for the preponderance
of A to T mutations in the second position 61st codon (T2)
of c-Ha-ras seen in papillomas after DMBA/TPA treatment.
One possibility would be increased frequency of T2 mutations
soon after DMBA initiation as a result of either selective DNA
adduct formation or repair. We have used the ‘i’ component
of the exponential best-fit lines to estimate that ~390 cells
possessed the T2 (CTA) mutation and a similar number
possessed the T3 (CAT) mutation in the original DMBA
treatment area. This area contains ~2.2x10^7 cells. The relative
initiation frequencies of T2 and T3 mutations (‘i’ = 0.305
and 0.301, respectively) may not be correct because of the variabil-
ity of the biological data, the sensitivity of the assays, and the
necessity to extrapolate points back in time. Therefore, we
cannot conclude that the T2 and T3 mutations are formed at
the same rate during the initiation process.

Another explanation for the abundance of T2 mutations in
papillomas is a selective growth advantage for cells with this
T2 mutation over those with a T3 mutation. The relative
strengths of the ‘g’ components from the statistical best-fit
lines for the T2 and T3 growth curves suggest a growth rate
during TPA promotion greater for the cells with the T2
mutation than for those with the T3 mutation. Because the
best-fit equations include initial mutation frequencies that are
no higher for T2 mutation than the T3, the effect of the
selective growth advantage reflected by the ‘g’ components
(0.552 for T2 compared to 0.276 for T3) could be sufficient
to explain both the increased T2 clonal expansion and the
preponderance of cells with the T2 mutation in papillomas.
Interestingly, this suggested difference of in vivo growth
rate corresponds closely to in vitro data using the relative
transforming activity of DNA for the 61st codon mutations.
Plasmid DNA that codes for the 61st codon leucine Ras (T2
mutation) produced 4.5 times more foci/ng DNA than DNA
that codes for the 61st codon histidine Ras (T3 mutation)
(22). However, all 61st codon mutant Ras proteins displayed
comparable reduced rates of GTP hydrolysis (22) showing the
complexity of the factors in this proliferation/differentiation
pathway. Our methods for sensitive measurements of clonal
development might be used in vitro to help define the complex
interactions of mutant Ras with upstream regulators as well
as downstream effectors particularly in co-culture where inter-
actions between cells more closely model the animal.

To detect the mutations before the appearance of tumors,
we needed sensitive and specific assays for each of these
mutations. The PCR/RFLP (23) method was chosen because
of its specificity. The assay was modified by the addition of a
nested PCR step (24). Another modification is the use of the
editing polymerase UlTma® (Perkin Elmer) under conditions
of low nucleotide concentration (4 µM) during the first stage
to amplify the wild-type and mutant c-Ha-ras alleles and
radiolabeling by incorporation of [3P]nucleotides in the second
stage with Stoffel® fragment polymerase (Perkin Elmer),
which has no 5’ to 3’ exonuclease (25). These improvements
create very complete, highly labeled copies of both alleles
with reduced background and reduced extraneous bands in the

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**Quantitation of early clonal expansion**

**Fig. 7.** Scatter plot of CAT (T3) mutant alleles of the 61st codon of
c-Ha-ras versus the harvest time in weeks after initiation with DMBA (50
µg) and weekly TPA (10 µg) promotion started one week after initiation. A
set of samples, representing the full time course and all treatment groups,
and a T3 plasmid standard curve were amplified and run together on a
1X TBE 8 5%/D-600 sequencing size gel. The same Stage I PCR product
that was assayed for T2 and G2 mutations entered a Stage II PCR
amplification with different primers. Digestion of the second stage 84 bp
product with NdeIII produced a 52 bp diagnostic band that was quantitated
by phosphorimaging, and converted to mutant copy number by comparison
to the standard curve. The log of mutant copies was plotted vs. harvest time
in weeks after DMBA initiation for the various treatments. Results shown
are derived from three independent experiments. Samples that have a
calculated mutant copy number of zero have been displaced to see all the
data points. The exponential best-fit line calculated by least squares is
shown.

**Discussion**

Historically, clonal expansion of ‘initiated’ cells has been the
working hypothesis and explanation for the effects of tumor
promoters during carcinogenesis (19). In this study, the clonal
expansion of cells carrying two different mutations in the 61st
codon of c-Ha-ras (T2 and T3) was quantitated with a nested
PCR/RFLP assay during TPA promotion in mouse skin initiated
with DMBA. The best fit lines of these data are exponential
curves as would be expected with the fraction of mutant
cells expanding in comparison to wild-type cells. This clonal
expansion was seen only with the group of mice that was both
initiated with DMBA and promoted with TPA, fitting the
model of mouse carcinogenesis would be expanding under the
influence of TPA to become clones of initiated cells seen as
frank papillomas (7).

The effects of TPA on epidermal basal cells in culture is
complex and includes both a stimulation of proliferation of one
subgroup of cells and an induction of terminal differentiation in
another subgroup of cells (8). Harvey (Ha) sarcoma virus-infected
cells when induced to differentiate are blocked at an
early stage of maturation and respond to TPA with a reversion
to a less mature state (9). Purified mutant Ras proteins have
been shown to have reduced GTPase activity that maintain the
Ras/GTP complex in a constantly activated form (20,21). Thus
TPA promotion, which results in clonal expansion of mutant
c-Ha-ras cells in the skin, could be characterized by disruption
of the normal signaling in the ras pathway, increased prolifera-

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lanes. Standard plasmid dilutions for the three mutations supplied a reference for converting diagnostic band intensity to number of mutant copies and allowed for comparisons between different mutations. Quantitating wild-type copies present in this nested PCR/RFLP assay proved to be a helpful index (26) to compensate for differences in PCR efficiency, quality or quantitation of the DNA as well as pipetting and loading differences.

False positive bands seen with samples from untreated mouse skin were a complicating factor in the nested PCR/RFLP assay for the CAG 61st codon mutation (G2). Predigestion of sample DNA with the diagnostic enzyme TaqI demonstrated that these products were formed during the PCR amplification. TaqI may have a lower level of sequence specificity than the restriction enzymes used to identify the other mutations. More likely these products represent the errors by Taq polymerase and by the 3' to 5' exonuclease proofreading enzyme UIIma.

PCR fidelity is affected by both insertion of a wrong base and further extension from that base. The fidelities of different polymerases vary in each step and under various conditions for various mismatches (27). A lack of extension from a mismatch or from a sequence related termination would produce a false positive product. These can be seen on gels, in lanes with and without digestion, for all three mutations at high cycle number and high specific activity (Figure 2). The presence of both diagnostic bands at much higher levels in the G2 assay suggests extension of a G misinsertion. In general G:T mismatches extend most efficiently, while A:A mismatches extend least efficiently (27). The G:T mismatch occurs in the G2 assay on the extension of the antisense strand of the wild-type allele. The less efficiently extended A:A mismatch causes less background as seen in the more sensitive T2 and T3 assays.

One would expect to see an almost equal level of the G2 mutation to the T3 mutation because of the near equal frequency of papillomas with those two mutations (6). We think that with greater sensitivity we may have detected the G2 mutation in the epidermis during the 12-week experiment. Various laboratories have reported 61st codon c-Ha-ras mutations CGA(G2) in tumors after promotion without initiation in Sencar animals (28,29). This supports the hypothesis that cells with this mutation clonally expand under promotion with TPA. Harvest times as long as 60 weeks and extended periods of TPA promotion of up to 30 weeks used in these 'promotion without initiation' experiments would allow time for expansion of G2 mutations formed at a very low 'spontaneous' rate. Significant differences in susceptibility to skin carcinogenesis between CD-1 mice used in our study and Sencar animals have been noted. Formation of the CCA 61st mutation during fetal development of Sencar animals has been hypothesized. Sencar animals may be defective in the repair of misinserted bases (29).

We feel that this assay might be useful to compare different carcinogens that create 61st codon mutations for their mutation rate by a comparison of the 'i' component of growth curves. This method can also be used to compare promoters to determine their relative strength by analysis of the 'g' component of the exponential curve. The nested PCR/RFLP method could be used in other carcinogenesis models where particular mutations are thought to expand under various promotion treatments or where different mutations have been suggested to act only during a particular stage, over particularly long periods of promotion, or where more than one promoter is thought to be effective. Initiation and promotion might be able to be estimated by the characteristics of the clonal expansion before tumors actually develop.

In summary, we have used a sensitive nested PCR/RFLP to quantitate CFA and CAT 61st codon mutant c-Ha-ras alleles in the early stages of mouse skin tumorigenesis using a DMB/TPA two stage protocol. Clonal expansion was shown for both the T2 and T3 mutation bearing cells during the 12 week experiment. TPA promotion appears to offer a sufficiently higher growth advantage to cells with a T2 mutation over those with a T3 mutation to account for the high frequency of the T2 mutation observed in papillomas formed by this method.

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References


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