Polymorphisms, but Lack of Mutations or Instability, in the Promotor Region of the Mitochondrial Genome in Human Colonic Tumors

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ABSTRACT

The region of the human mitochondrial D-loop has been sequenced from DNA of colonic tumors and paired normal colonic tissue to determine if mutations in the promoters for the heavy or light strands are responsible for the decrease in mitochondrial gene expression present in colonic tumors. No mutations were detected in the colonic tumors, but new polymorphisms, including a sequence analogous to CA microsatellites in genomic DNA, were revealed. These polymorphisms are restricted to positions within the D-loop which are not essential for accurate and efficient in vitro mitochondrial transcription. Thus, these data confirm the boundaries of the functional heavy and light strand promoters determined by in vitro assays. Further, although some of the tumors investigated show genomic microsatellite instability similar to that reported for colon tumors, the CA polymorphic region in the mitochondrial D-loop does not show coincident instability in the tumors. Therefore, as in yeast, there may be both a mitochondrial and a nuclear enzyme responsible for mismatch repair, with only the latter involved in generation of instability in some human colon cancers. In summary, our data do not find any structural alterations in the D-loop region of the human mitochondrial genome encompassing the heavy and light strand promoters which can account for the decreased expression of the mitochondrial genome in colonic tumors.

INTRODUCTION

The mitochondria of colonic tumors are aberrant in both structure and function (1, 2). Associated with this is decreased expression of the mitochondrial genome in these tumors. We reported that the mt\(^3\) gene which encodes the third subunit of the mt localized enzyme cytochrome oxidase, COXIII, is decreased in expression in human colonic tumors as compared to the normal colonic mucosa (3). This is paralleled by decreased cytochrome c oxidase activity in colonic tumors compared with normal mucosa (4, 5). We have also reported that specific short-chain fatty acids which can induce colonic carcinoma cell differentiation (e.g., Refs. 6 and 7) and apoptosis (8, 9) in vitro also induce the expression of COXIII, as well as COXI, and elevate cytochrome oxidase activity in isolated mitochondria (10).

These modulations in mt gene expression in transformed and differentiated colonic epithelial cells may reflect more general changes in expression of a number of mt genes and enzymes. Two additional genes encoded by the mt heavy (H) strand (COXII and ND3) are decreased in expression in the flat mucosa of patients with familial polyposis or hereditary non-polyposis colon cancer compared to low-risk flat mucosa. This substantial decrease in mt gene expression in the flat mucosa of genetic risk patients may explain why polyps from such patients show elevated expression when compared to the adjacent mucosa (11) but decreased expression when compared to the mucosa of patients at low risk (3). Further, induction of differentiation of colonic carcinoma cells in culture with specific short-chain fatty acids elevates mt rRNA and ND3 expression on the H-strand in addition to COXI, -II, and -III and ND6 on the L-strand. Other inducers of colonic cell differentiation also elevate expression of a number of H- and L-strand mt genes (12).

Previous studies have shown that mitochondrial DNA is modified by chemical carcinogens to a far higher degree than is nuclear DNA (13–15), and compared to the nuclear genome, there is a general deficiency of DNA repair in mitochondria (e.g., Ref. 16). Therefore, the purpose of this study was to determine if there was a structural basis (i.e., mutation) for the decreased mt gene expression in colonic tumors. We approached this question by sequencing a portion of the mt D-loop encompassing the promoters for the H- and L-strand in DNA isolated from tumors and adjacent mucosa of 24 patients with colonic cancer. We found that compared to normal tissue of the same patients, tumors lacked mutations in this region. Furthermore, we document the presence of previously unreported polymorphisms, including variation in a simple CA repeat analogous to nuclear microsatellite sequences (17, 18), in this area of the mitochondrial genome. The restriction of these polymorphisms to nucleotides just flanking the reported binding sites for the major mitochondrial transcription factor mtTF1 supports the definition of the boundaries of these regulatory elements based on in vitro data (19–21) and suggests that the polymorphisms are not functionally relevant. Finally, we found that some of the tumors in this set exhibit instability in a CA genomic microsatellite but no coincident instability in the polymorphic CA sequence in the mt D-loop. This is of interest since such instability in some human colon mucosa (22–24) has been speculated to lead to altered gene expression (23, 24). Further, we have found instability in nuclear loci which are also elevated in expression in colonic mucosa at high risk for tumor development. Thus, no evidence is found for alterations in the mitochondrial H- and L-strand promoters which could account for the decreased expression of the mt genome in colonic tumors.

MATERIALS AND METHODS

DNA was isolated as reported (25). PCR amplifications (26) were done in a reaction mixture consisting of 50 mM KCl, 10 mM Tris (pH 9.0) at 25°C, 0.1% Triton X-100, 2.5 mM MgCl\(_2\), 100 mM concentrations of each deoxynucleotide triphosphate, 0.5 μg of DNA, 10 pmol of each primer, and 2.5 units of Taq polymerase (Promega) in a final volume of 50 μl. Samples were denatured at 95° for 5 min and then cycled 40 times at 95° for 1 min, 55° for 1 min, and 72° for 1 min.

For analysis of the mitochondrial D-loop sequence, amplification was done with the following primers: sense, 5'CTAACACCGCCTCAACCCAG; antisense, 5'GGGGTGATGTGAGCCCGTCF. The amplified DNA was cloned into pT7Blue (Novagen). DNA from >100 pooled clones were sequenced by dideoxy chain termination (27) using the primer 5'TTGGAGGGAGTGAGCCCTCATC encoded in the amplified mitochondrial DNA.

For analysis of microsatellite instability, the (CA)$_y$ repeat located 222 base pairs upstream of the TATA box of the apolipoprotein D gene (28), was

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3 The abbreviations used are: mt, mitochondrial; PCR, polymerase chain reaction; HNPCC, hereditary non-polyposis colonic cancer.

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5 B. G. Heerdt and L. H. Augenlicht, unpublished observations.

6 J. Chen et al., submitted for publication.

7 J. Chen et al., submitted for publication.

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10 J. Chen et al., submitted for publication.
amplified using the conditions above with the following primers: sense, 5'-CATGTGGCACACGTCTGGAAA; antisense, TCCTCAGACGT-GCTCCTGAAGAAG. The PCR reactions included 12.5 μCi [35S]ATP (NEN; 1.4 Ci/mm), and the products were analyzed on standard sequencing gels. The mt D-loop region was similarly analyzed for instability using the primers described above.

RESULTS AND DISCUSSION

We amplified, cloned, and sequenced the D-loop region from nucleotide 371 through approximately 570 (numbering as in Ref. 29) of human mitochondrial DNA from paired samples of colon tumor and normal colonic tissue from 24 different patients. For each tumor, the sequence obtained was identical to that from the corresponding normal tissue. These results are summarized in Fig. 1.

Besides the lack of mutations in the tumor DNA, two additional conclusions can be reached from the data in Fig. 1. First, there are several polymorphic sites in this region of the D-loop which encompass the H- and L-strand promoters (HSP and LSP, respectively). These data fall into three groups. For the first 9 pairs of samples (Fig. 1), the sequence was identical to the published sequence determined from a combination of human placenta and HeLa cell DNA (29) and to a placental DNA sample sequenced by us (not shown). The next 8 pairs of samples contained base substitutions. The last group contained deletions or additions in a CA repeat found 5 times in the published sequence at position 514. The frequencies of these polymorphisms are summarized in Table 1.

The locations of the polymorphisms in relation to functional elements in this region of the mitochondrial genome are also depicted in

Fig. 1. Position of polymorphisms in the human mitochondrial D-loop in relation to known promoter elements. Some published data are based on a CA polymorphism at 514 of 4 repeats, rather than 5 (e.g., Refs. 19 and 31). Numbering has been adjusted where necessary to conform to the baseline sequence in Ref. 29. [i.e., (CA), at position 514, with the start sites for H- and L-strand transcription at 561 and 407, respectively]. Arrows, initiation sites and direction of transcription of the H- and L-strands of the human mitochondrial genome. @, boundaries of the promoter elements necessary for accurate and efficient in vitro transcription as defined in Ref. 31. † boundaries of the functional promoter as subsequently defined in Ref. 19. The baseline sequence of HeLa/placenta (Ref. 29 and our data, not shown) is shown only for those nucleotides which vary in any of the tumor (T, ordinate) or normal (N) DNA samples investigated. HSP, H strand promoter; LSP, L strand promoter.

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The transcripational start sites and HSP and LSP boundaries are shown at the top (numbering as in Ref. 29; see Fig. 1 legend). The element necessary for accurate initiation of H-strand transcription has been defined by deletion analysis as nucleotides 545–568, but since efficiency of transcription was modulated by deletions further upstream, the promoter for the wild type level of in vitro transcription is depicted in Fig. 1 as 501–568 (30, 31). Similarly, the analogous element necessary for accurate L-strand transcription is 435–391, but modulation of efficiency of transcription further extends the region required for wild type in vitro transcription up to 463 (31). This general organization of the promoters was focused by linker substitution analysis which narrowed the regions required for in vitro transcription to 527–568 for the heavy strand and 446–396 for the light strand (19), shown as shaded regions in Fig. 1. These more limited boundaries to the functional domains which bind mtTF1 (20), a nuclear encoded mitochondrial transcription factor (32, 33), and to sequences necessary for accurate transcriptional initiation at 561 and 407 for the heavy and light strands, respectively (21).

It is clear from Fig. 1 that almost all of the naturally occurring polymorphic positions fall outside these regions defined as critical for accurate and efficient transcription (19). The one exception is an A to G transition at position 533 in patient 87-4002. However, while the C at 530 (~31 from the initiation site) is required for in vitro transcription, nucleotides 534–536 could be varied with minimal effect (21). Effects of variation at 533 were not reported, but the data of Fig. 1 predict that this position is also not critical for in vitro transcription. Thus, since the polymorphisms fall outside the functionally significant regions of the promotor, it is unlikely that they play a role in tumor development.

Table 1 Polymorphisms in the human mitochondrial D-loop

<table>
<thead>
<tr>
<th>Position</th>
<th>Published</th>
<th>Polymorphism</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>462</td>
<td>C</td>
<td>T</td>
<td>1/24</td>
</tr>
<tr>
<td>489</td>
<td>T</td>
<td>C</td>
<td>1/24</td>
</tr>
<tr>
<td>497</td>
<td>C</td>
<td>T</td>
<td>5/24</td>
</tr>
<tr>
<td>499</td>
<td>G</td>
<td>A</td>
<td>1/24</td>
</tr>
<tr>
<td>508</td>
<td>A</td>
<td>G</td>
<td>1/24</td>
</tr>
<tr>
<td>512</td>
<td>A</td>
<td>C</td>
<td>1/24</td>
</tr>
<tr>
<td>514</td>
<td>(CA)$_3$</td>
<td>(CA)$_4$</td>
<td>7/24</td>
</tr>
<tr>
<td>533</td>
<td>A</td>
<td>G</td>
<td>1/24</td>
</tr>
</tbody>
</table>

* As in Ref. 29.

* Number of patients differing from published sequence/number assayed.

Although the sequencing data summarized in Fig. 1 did not show any evidence of sequence instability in the mt D-loop of the 24 tumors, instability was investigated in greater detail. Since tumors in HNPCC individuals tend to occur on the right side, 10 tumors from the ascending colon were investigated for genomic instability at a microsatellite upstream of the apolipoprotein D gene (28). Three tumors showed clear evidence of tumor-specific instability, seen as additional minor bands shown in Fig. 2, top. These additional bands were reproducible, and these same tumors showed instability at other loci as well.7 Fig. 2, top, shows three additional tumors for which no instability was seen.

These tumors were also analyzed for mt mutations (Fig. 1), and no evidence of instability in the mt CA repeat was seen. The PCR products of the amplified D-loop DNA of these tumors were then analyzed on a sequencing gel for the presence of more subtle instability. Two bands were seen, corresponding to the H- and L-strands, as indicated in Fig. 2B. This was determined by both the differences in relative incorporation of dCTP into the strands and end-labeling of the H- and L-strand-specific primers (not shown). In no case did we see evidence for length heterogeneity in the PCR product (Fig. 2B), unlike the additional bands seen for the nuclear locus (Fig. 2A). Thus, the instability in these tumors appears restricted to the nuclear genome. This is consistent with the fact that in yeast, mt mismatch repair is not carried out by the MSH2 gene product, the homologue of an HNPCC gene on chromosome 2 (34), recently identified as the human homologue of the yeast MSH2 gene (35), which functions in DNA mismatch repair.

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Footnotes:

7 J. Chen and L. H. Augenlicht, submitted for publication.
genome may encode separate enzymes for mismatch repair of mitochondrial and nuclear DNA, and only the latter are mutated in some HNPC families (35).

In summary, our data suggest that despite the higher rate of chemical modification of the mitochondrial genome by carcinogens (13–15) and despite the relative deficiency of repair of the mitochondrial genome (16), mutations are not seen in human colonics tumors in the mt transcriptional promoter regions as compared to the normal mucosa. Furthermore, instability is not detected in a CA polymorphic genome (16), mutations are not seen in human colonic tumors in the mitochondrial and nuclear DNA, and only the latter are mutated in expression. Factors or altered posttranscriptional regulation of mitochondrial gene repeat in this region. Therefore, the coordinate decrease in mitochondrial transcriptional promotor regions as compared to the normal mucosa. Heerdt, B. O., and Augenlicht, L. H. Effects of fatty acids on expression of genes 15) and despite the relative deficiency of repair of the mitochondrial genome. Modica-Napolitano, J. S., Steele, G. D., and Chen, L. B. Aberrant mitochondria in two human colon cancer cell lines. Cancer Res., 49: 3369–3373, 1989.


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