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Molecular Cloning of the Genes Suppressed in RVC Lymphoma Cells by Topoisomerase Inhibitors

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Etoposide is a topoisomerase II inhibitor that induces DNA cleavable complex and has been used as an antitumor drug. We isolated two genes that were transcriptionally suppressed at an early stage of incubation in etoposide-treated RVC lymphoma cells, using modified PCR-based subtractive hybridization. Sequencing revealed that one of these genes, which was approximately 1.7 kb and which encoded a protein of 320 amino acids, was identical to hnRNP A1. The other was a novel gene of about 2.2 kb encoding a protein of 469 amino acids. These genes were also down-regulated in the cells incubated with camptothecin, a topoisomerase I inhibitor that induces DNA single strand breaks, but not in those exposed to ICRF-154, a topoisomerase II inhibitor that does not induce DNA cleavable complex formation. These results suggest that the early down-regulation of these genes contributes to the cytotoxicity of the topoisomerase inhibitors that induce DNA cleavage.

DNA topoisomerases catalyze topoisomerization and are involved in regulating chromosome structure, DNA replication, transcription and mitosis in eukaryotes. Topoisomerases have been divided into at least two classes based on their mechanism of action (1-3). The type I enzyme catalyzes by transiently cleaving one DNA strand for the passage of another and appears to be the major cellular activity in the removal of supercoils generated by processes such as replication and transcription. The type II enzyme catalyzes the transient breakage of double-stranded DNA and the transport of another DNA double helix through the break before its sealing. This enzyme is essential for disentangling DNA during condensation and the mitotic and meiotic segregation of chromosomes. Recently, DNA topoisomerases have been identified as the molecular targets of a number of antitumor drugs (4, 5). Camptothecin is a topoisomerase I inhibitor that induces DNA single strand breaks (6). Etoposide is a topoisomerase II inhibitor that induces ''cleavable complex'', the stabilized reaction intermediate (7). Many epipodophyllotoxins, isoflavonoids and intercalaters such as amsacrines, anthracyclines and actinomycins are included in this class. Bis(2,6-dioxopiperazine) derivatives (8) including ICRF-154 are topoisomerase II inhibitors that do not induce ''cleavable complex''. However, the mechanism of topoisomerase cytotoxicity has not been clearly elucidated. It is likely that the changes in the topological state of chromatin DNA induced by topoisomerase inhibitor alter the expression of certain genes, resulting in cytotoxicity. Therefore, we investigated the genes of which the expression is altered in RVC lymphoma cells exposed to topoisomerase inhibitors. In this study, we describe the cloning and characterization of 2 genes of which the expression was reduced by etoposide and camptothecin.

MATERIALS AND METHODS

Cell lines and culture conditions. The radiation leukemia virus-induced leukemia RVC cells(9) were routinely cultured in RPMI 1640 medium containing 10% fetal bovine serum, 50 mM 2-mercaptoethanol, 10 mM HEPES, 100

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Abbreviation: G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

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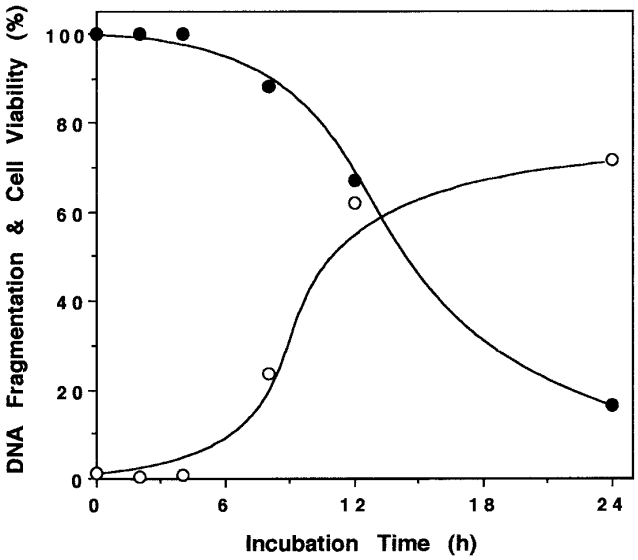

FIG. 1. Time-courses of etoposide-induced DNA fragmentation and cell viability. RVC cells were incubated with 5 μM etoposide. DNA fragmentation (○) and cell viability (●) were determined at the indicated times as described in ''MATERIALS AND METHODS''. Data represent mean values of triplicate determinations.

U/ml penicillin and 100 μg/ml streptomycin. Incubation proceeded at 37°C in a humidified incubator under a 5% CO_2 atmosphere.

DNA fragmentation and cell viability. After RVC cells were incubated with 5 μM etoposide, fragmentation assay of chromatin DNA was proceeded as described previously (10) and cell viability was determined by the trypan blue exclusion test.

cDNA cloning. Poly(A)$^+$ RNA was prepared from RVC cells cultured with or without etoposide at 5 μM for 4 h as described previously (11). The cDNA was subtracted according to the method of Sive and St. John (12). The single strand cDNA, which was synthesized from 1 μg of poly(A)$^+$ RNA from untreated RVC cells, was hybridized with biotinylated mRNA from RVC cells treated with 5μM etoposide. The subtracted cDNA or unhybridized cDNA was purified by phenol extraction and then tailed with dCTP using terminal deoxynucleotidyl transferase (TOYOBO, Osaka). The tailed cDNA was amplified by PCR using the following primers: primer #1, 5′-GCGAAAGCTTG_{15}-3′ (HindIII site); primer #2, 5′-CGAGGAATTCT_{30}-3′ (EcoRI site). The temperature protocol was: 40 cycles of 94°C/1 min; 50°C/2 min; 72°C/3 min. The cDNA was digested with EcoRI and HindIII (TOYOBO), ligated into pUC118 (TAKARA, Kyoto), and transformed into MV1184. Clones representing genes transcriptionally suppressed by etoposide were identified by differential colony hybridization (13) using the cDNA reverse-transcribed from mRNA of RVC cells with or without etoposide as a probe. Colonies that hybridized with the probe from nontreated RVC cells were isolated and analyzed. We obtained a full length cDNA using RACE PCR (14).

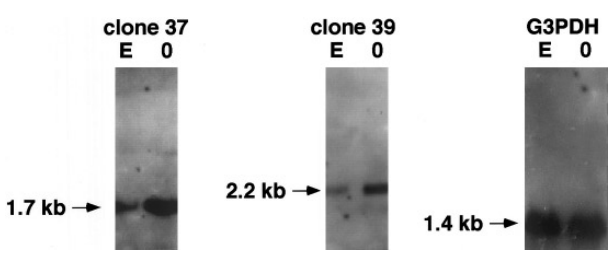

FIG. 2. Northern blot analysis of mRNA from RVC cells incubated with etoposide. RVC cells were incubated with (lane E) or without (lane 0) 5 μM etoposide for 4 h, then poly(A)$^+$ RNA was prepared and blotted. The blotted membrane was hybridized with the cDNA clones 37, 39 and G3PDH cDNA as described in ''MATERIALS AND METHODS''. Arrows represent transcript size.

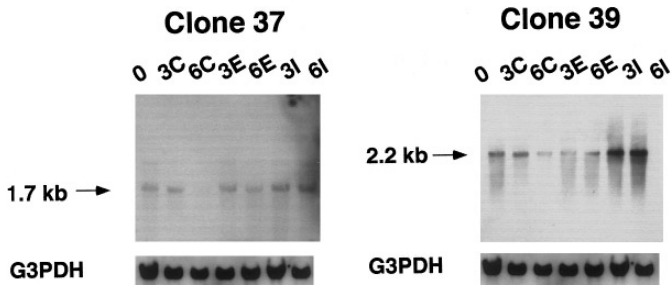

FIG. 3. Effect of camptothecin, etoposide and ICRF-154 on the expression of clone 37 and 39 transcripts. The poly(A)$^+$ RNA was prepared and blotted onto a membrane and hybridized with the cDNA clones 37, 39 and G3PDH cDNA as described in ''MATERIALS AND METHODS''. RNA samples: lane 0, control RVC cells; lanes 3C and 6C, RVC cells treated with 5 μM camptothecin for 3 h and 6 h, respectively; lanes 3E and 6E, RVC cells treated with 5 μM etoposide for 3 h and 6 h, respectively; lanes 3I and 6I, RVC cells incubated with 20 μM ICRF-154 for 3 h and 6 h, respectively. Arrows indicate transcript size.

Northern blot analysis. Northern blot analysis was proceeded as described previously (11). The DNA probes were cDNA inserts and G3PDH cDNA (Clontech Laboratories, Palo Alto, CA).

DNA sequencing. DNA was sequenced using the Taq Dye Primer Cycle Sequencing Kit, the Taq Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a thermal cycler. An automated DNA sequencer (Applied Biosystems model 373A) was used to determine the nucleotide sequence, which was analyzed by the DNASIS-Mac software (Hitachi Software Engineering, Yokohama, Japan). The DDBJ (DNA Data Bank of Japan) sequence databases were searched for similar nucleotide sequences using the FASTA program.

RESULTS AND DISCUSSION

Topoisomerase inhibitors have been shown to modulate differentiation (15), indicating that they are involved in the control of gene expression. We showed that inhibiting topoisomerase activity and changing the topology of chromatin DNA were important for the cytotoxicity induced by etoposide in mouse thymocytes and that these events occurred within a few hours after adding etoposide to the cells (16). When RVC cells were incubated with etoposide, apoptosis was induced as in thymocytes (17). DNA fragmentation increased and cell viability decreased after 8 h incubation (Fig. 1). These results suggest that the cellular event(s) essential for cell death occurred within a few hours after exposure as seen in etoposide-treated thymocytes. We then tried to identify the genes involved in cytotoxicity, and whose expression was altered soon after adding etoposide. We isolated clones 37 and 39, which were suppressed. We then performed Northern blot analysis using each cDNA insert of these clones (Fig. 2). The mRNAs of clones 37 and 39 were about 1.7 and 2.2 kb in size, respectively. Both transcripts were reduced about 2-fold within 3 h after etoposide was added, reaching about a 10 fold reduction after 6 h (Fig. 3). The reduction was observed before the DNA fragmentation occurred dominant (Fig. 1). The transcripts were reduced even in the presence of cycloheximide, suggesting that the cDNA clones 37 and 39 were transcriptionally reduced independently of protein synthesis.

The entire sequence of these cDNAs was determined and searched for similarity using the FASTA program. As shown in Fig. 4, the complete transcript of the clone 37 consisted of 1735 bases encoding 320 amino acids, that was identical to hnRNP A1 (18). The nucleotide sequence had 93% homology to rat helix-destabilizing protein / hnRNP A1, and a shorter transcript with a deletion of 159 nucleotides corresponding to 53 amino acids (amino acids #251-302) was generated at the level of one twentieth of the whole transcript by RT-PCR. The deleted nucleotides corresponded to the 8th exon in human hnRNP gene (19). Buvoli et al. reported that hnRNP A1 is encoded by a multigene family yielding multiple mRNA isoforms

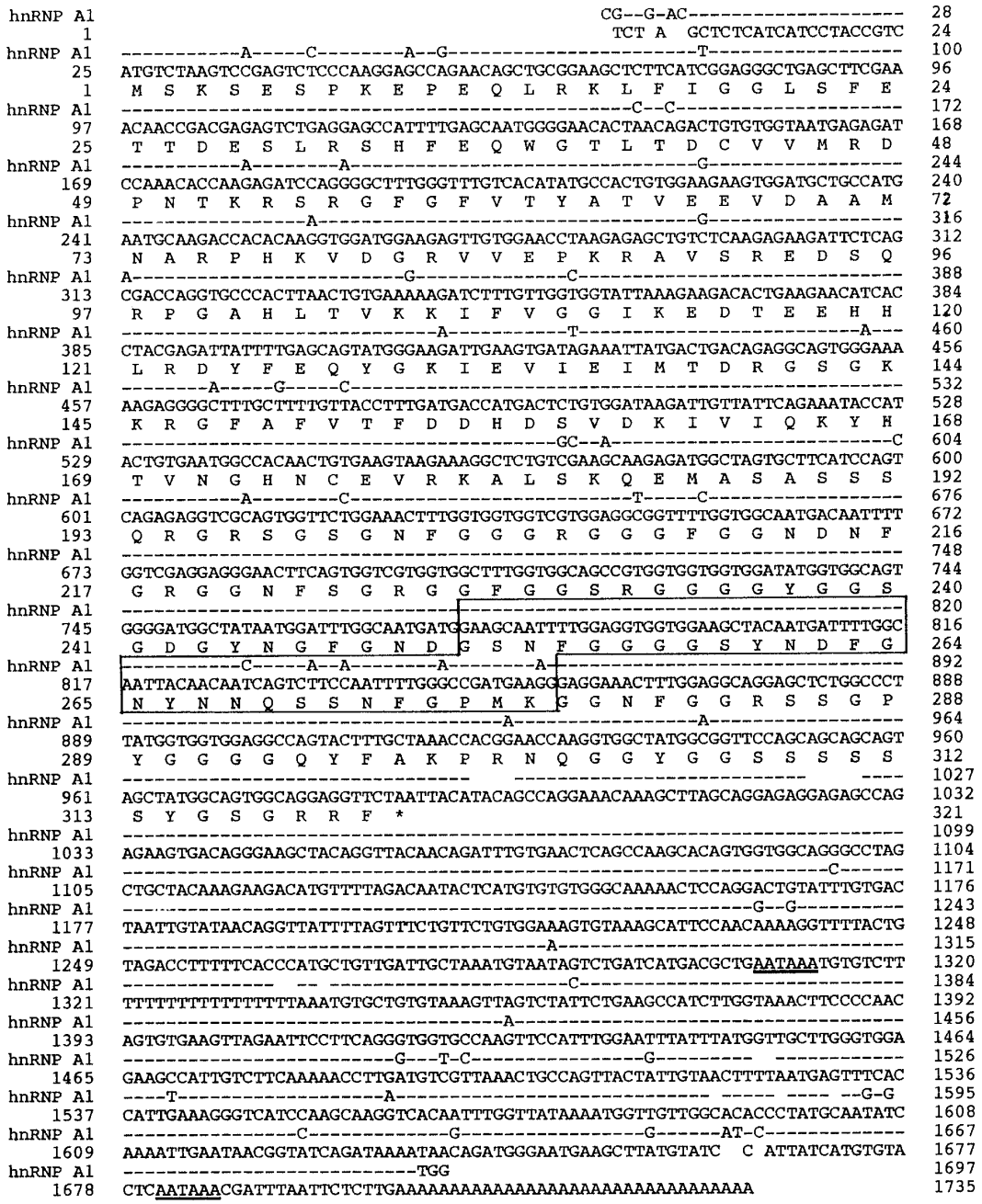

FIG. 4. Nucleotide and deduced amino acid sequences of cDNA clone 37. Nucleotide sequence of clone 37 compared with rat helix destabilizing protein/hnRNP A1. Identical nucleotides are shown by dotted lines in the rat hnRNP A1 cDNA sequence. The nucleotide sequences missing in the shorter transcript are boxed. The predicted amino acids sequence is shown under the nucleotide sequence and the positions of nucleotide and amino acid residues are indicates at the sides. In-frame stop codons are indicated asterisks. A potential polyadenylation signal, AATAAA, is underlined. The nucleotide sequence data of complete and shorter cDNA clone 37 have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers D86728 and D86729, respectively.

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   1                                                                CTAGCGGCGGCGGC   14
  15  CCGAGGGGATCCTCAAGGAAGACTTGCATTTAGTAAGTTGTGTATAATCAGTGCAAGCGAAATTAAGGAAAA   86
  87  ATGGATATAGAAAATGAGCAGACACTGAATGTGAACCCCACTGACCCTGACAATTTAAGCGACTCTCTCTTT  158
   1   M  D  I  E  N  E  Q  T  L  N  V  N  P  T  D  P  D  N  L  S  D  S  L  F   24
 159  TCTGGAGATGAAGAAAATGCTGGCACTGAAGAAATAAAGAATGAAATAAATGGAAATTGGATTTCCGCATCT  230
  25   S  G  D  E  E  N  A  G  T  E  E  I  K  N  E  I  N  G  N  W  I  S  A  S   48
 231  ACTATTAATGAAGCTAGAATCAATGCAAAAGCCAAAAGACGACTGCGGAAAAATTCATCCCGGGACTCTGGC  302
  49   T  I  N  E  A  R  I  N  A  K  A  K  R  R  L  R  K  N  S  S  R  D  S  G   72
 303  CGAGGAGACTCAGTCAGTGACAATGGAAGTGAAGCGGTTAGAAGTGGAGTTGCTGTGCCCACCAGTCCAAAA  374
  73   R  G  D  S  V  S  D  N  G  S  E  A  V  R  S  G  V  A  V  P  T  S  P  K   96
 375  GGAAGGTTGCTAGATAGGCGGTCCAGATCTGGGAAAGGAAGGGGGCTGCCAAAGAAAGGTGGTGCAGGCGGC  446
  97   G  R  L  L  D  R  R  S  R  S  G  K  G  R  G  L  P  K  K  G  G  A  G  G  120
 447  AAGGGTGTCTGGGGCACACCTGGACAGGTGTATGATGTGGAAGAGGTGGATGTGAAAGATCCAAACTATGAT  518
 121   K  G  V  W  G  T  P  G  Q  V  Y  D  V  E  E  V  D  V  K  D  P  N  Y  D  144
 519  GACGACCAGGAGAACTGTGTTTATGAAACTGTAGTTTTGCCCCTGGATGAGACCGCATTTGAGAAGACTCTA  590
 145   D  D  Q  E  N  C  V  Y  E  T  V  V  L  P  L  D  E  T  A  F  E  K  T  L  168
 591  ACACCAATTATACAGGAATACTTTGAGCATGGAGATACAAATGAAGTTGCGGAGATGTTAAGAGACTTAAAC  662
 169   T  P  I  I  Q  E  Y  F  E  H  G  D  T  N  E  V  A  E  M  L  R  D  L  N  192
 663  CTTGGGGAGATGAAGAGTGGCGTGCCGGTGTTGGCAGTGTCCTTAGCCTTGGAGGGGAAGGCCAGCCACCGG  734
 193   L  G  E  M  K  S  G  V  P  V  L  A  V  S  L  A  L  E  G  K  A  S  H  R  216
 735  GAGATGACATCCAAGCTGCTTTCTGACCTTTGCGGGACGGTGATGATCACAAATGACGTGGAAAAGTCATTT  806
 217   E  M  T  S  K  L  L  S  D  L  C  G  T  V  M  I  T  N  D  V  E  K  S  F  240
 807  GACAAGTTGCTGAAGGATCTCCCTGAGCTAGCCTTGGACACTCCTAGGGCACCGCAGTTGGTGGGCCAGTTT  878
 241   D  K  L  L  K  D  L  P  E  L  A  L  D  T  P  R  A  P  Q  L  V  G  Q  F  264
 879  ATTGCTAGAGCTGTTGGAGATGGAATCTTATGTAATACCTATATCGATAGTTACAAAGGAACTGTAGATTGT  950
 265   I  A  R  A  V  G  D  G  I  L  C  N  T  Y  I  D  S  Y  K  G  T  V  D  C  288
 951  GTACAGGCTCGAGCTGCTCTGGATAAGGCTACTGTGCTCCTGAGTATGTCCAAAGGCGGGAAGCGGAAAGAC 1022
 289   V  Q  A  R  A  A  L  D  K  A  T  V  L  L  S  M  S  K  G  G  K  R  K  D  312
1023  AGTGTGTGGGGATCTGGAGGCGGGCAACAGCCTGTCAATCACCTTGTTAAAGAGATTGATATGCTGCTTAAA 1094
 313   S  V  W  G  S  G  G  G  Q  Q  P  V  N  H  L  V  K  E  I  D  M  L  L  K  336
1095  GAGTATTTACTCTCTGGAGATATATCTGAAGCTGAACACTGCCTTAAGGAACTGGAAGTACCTCATTTTCAC 1166
 337   E  Y  L  L  S  G  D  I  S  E  A  E  H  C  L  K  E  L  E  V  P  H  F  H  360
1167  CACGAGCTTGTATATGAAGCCATTATAATGGTTTTAGAGTCAACTGGAGAAAGTGCATTCAAGATGATCTTA 1238
 361   H  E  L  V  Y  E  A  I  I  M  V  L  E  S  T  G  E  S  A  F  K  M  I  L  384
1239  GATTTATTAAAATCCTTGTGGAAGTCTTCTACTATTACCATAGACCAAATGAAAAGGGGCTATGAGAGAATT 1310
 385   D  L  L  K  S  L  W  K  S  S  T  I  T  I  D  Q  M  K  R  G  Y  E  R  I  408
1311  TACAATGAAATCCCAGACATTAATCTGGATGTCCCGCACTCATACTCTGTTCTTGAGAGATTTGTGGAGGAA 1382
 409   Y  N  E  I  P  D  I  N  L  D  V  P  H  S  Y  S  V  L  E  R  F  V  E  E  432
1383  TGTTTTCAGGCTGGAATAATTTCCAAACAACTCCGTGATCTTTGTCCATCAAGGGGAAGAAAGCGTTTTGTA 1454
 433   C  F  Q  A  G  I  I  S  K  Q  L  R  D  L  C  P  S  R  G  R  K  R  F  V  456
1455  AGTGAAGGAGATGGAGGCCGTCTTAAACCTGAGAGCTACTGAGCACAGCAACTCTTACAGTCTTAGGTGTTA 1526
 457   S  E  G  D  G  G  R  L  K  P  E  S  Y  *                               470
1527  CCAAGAACAGATCTCAACTGTAAGAGTTGTCAGTACAGGTTTTCTCTTCCTTTTGTTTTTGTTATTGTTTTT 1598
1599  TTTTTTTTTTTTTTTTTTTTAAAGAATTTGTTTTGGGTACAAGGCATTTCTAAAATTTTATAAACTTAAGTTT 1670
1671  AATGGGATTTTTTGAAGGATTTTCTTTCTTTTTCTTTTTCTTTCTTTTTTTTTGAGGGGAAATTAATGGAGG 1742
1743  GACAGAAGAGGAACCACCGAAGTGTGGGTGTTCTGATAAGCTACTTTCTAAGTGCCATGTTTAGGACCTGAT 1814
1815  CATTCCAAGTTTCACGTTCATGTATGACTGCCGCTCCTTTCTTTCAAGGACAGTGTTTTTTGTAGTAAAATC 1886
1887  ACTGGTTTATTCAAAGCTTTAGTTAGGGGTGAGTCAAGCTACTAAACCCCATGTTGGCTGCTGCTGTGGAAA 1958
1959  TGCTGTCCTTTGAGAGTAAACACACACACACACACACACACACTTTTTGTTTTAAAGAATTTTTAAAAAAAC 2030
2031  GAGTTAGTCATGAGACTTTTTCATCTTTCCAGGGAATATATTGATTGGTCTTAAATATTAGACAGTTAAGTA 2102
2103  AATGGTGGCTGGAACATCTATTTTTCTACAAAACTGGAAAAACGAACCCGGTTCTACAAGAATGTACAGCAA 2174
2175  AATAAAACATGTGAAACACTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                   2229
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FIG. 5. Nucleotide and deduced amino acid sequences of cDNA clone 39. The predicted amino acids sequence is shown under the nucleotide sequence and the positions of nucleotide and amino acid residues are indicates at the sides. In-frame stop codons are indicated by asterisks. A potential polyadenylation signal, AATAAA, is underlined. The nucleotide sequence data of cDNA clone 39 have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D86344.

(20). This shorter transcript may be an mRNA isoform formed by alternative splicing. The hnRNP A1 counteracts the activity of the SF2/ASF factor in regulating the splicing of selected transcripts (21). Moreover, hnRNP A1 affects mRNA stability by binding to splice sites (22). These facts suggest that the etoposide-induced alteration of the topology of chromatin DNA modulated the level of hnRNP A1, resulting in cytotoxicity.

Figure 5 shows that the complete transcript of clone 39 consisted of 2229 bases, and it encoded a protein of 469 amino acids of which the first methionine was determined by the rule described by Kozak (23). The predicted amino acid sequence showed no significant homology to known other proteins. The calculated molecular weight and isoelectric point of the novel gene product were 51739 and 4.9, respectively. However, its physiological function remains unknown.

The topoisomerase II activity was determined as described previously (17). Etoposide at 5 μM inhibited the topoisomerase II activity to about 60.8 % of the control. ICRF-154 at 20 μM inhibited the activity to the same level as 5 μM etoposide in vitro (59.6 %). However, in ICRF-154-treated cells, the expression of both hnRNP A1 and the novel gene was not reduced as it was in cells exposed etoposide (Fig. 3). These results suggest that the inhibition of the enzyme activity is not directly related to the suppression of the genes and that the antitumor effect of ICRF-154 differs from that of etoposide. Camptothecin is a topoisomerase I inhibitor, but it suppressed the expression of the genes like etoposide (Fig. 3). Considering that camptothecin and etoposide induce DNA single and double strand breakage, respectively, camptothecin may share the same cytotoxic process with etoposide and the drug-induced DNA cleavage may play a key role in the reduction of these genes and cytotoxicity. Nelson and Kastan reported that topoisomerase inhibitors rapidly triggered p53 tumor suppressor protein elevation, then induced apoptosis in tumor cells (24). Elucidation of the relationship between the elevation of p53 and the reduced expression of the gene which we isolated should be of interest.

In summary, we isolated two cDNA clones and determined their entire nucleotide sequence. Their expression was suppressed within a few hours in RVC cells incubated with topoisomerases inhibitors that cleave DNA. One gene was identical to hnRNP A1 and the other was novel. We are now studying the mechanism of suppression of these genes and its role in cytotoxicity, as well as the physiological function of the novel gene.

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