Characterization of RNA with unusual electrophoretic mobility from tissues of patients with Crohn's disease

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Abstract RNAs from tissues of patients with Crohn's disease that migrate off the diagonal in a two-dimensional gel electrophoresis system were partially characterized. One of the RNA species was a discrete cleavage product of region V2-9 of 28S rRNA; another is a conformer or variant of 5.8S rRNA; and a third is a mixture of unidentified fragments with mobility similar to that of 7S RNA. The yield of these species from resected tissue and their visualization by silver staining was very sensitive to the details of the preparative procedure. No evidence of viroid-like RNA was found within the range of molecular sizes (<7S) that we examined.

Key words: rRNA, 28S; Endoribonuclease; Cell death; Human intestinal tissue

1. Introduction

Crohn's disease (CD) is characterized by chronic inflammation of the gastrointestinal tract. The etiology of CD is unknown although the influence of a number of factors, such as diet, other environmental factors, genotype underlying the auto-immune response or an unidentified infectious entity, has been suggested [1,2].

A search has been made for novel small RNAs (i.e. viral or viroid RNA) in tissues from patients with CD but no differences were seen in comparisons of RNA isolated from diseased and control tissues after one-dimensional gel electrophoresis [3]. Pechan et al. [4] used a highly sensitive silver-staining method coupled with two-dimensional gel electrophoresis, which was originally developed for identification of viroid RNA [5], to search for putative novel RNAs. Using this method, they detected RNA with unusual electrophoretic mobility in diseased tissue but not in control tissue. This result, consistent with the detection of circular RNA and taken together with the apparent size of the RNA, led Pechan et al. [4] to suggest that viroid-like RNA might exist in CD tissue. However, they undertook no further analyses.

We confirmed the observations of Pechan et al. [4] and subsequently characterized three different RNA species that migrated off the diagonal in a two-dimensional electrophoretic system. None of these RNAs had the primary sequence or physical properties associated with viroid RNA. Their detection was very sensitive to the details of the RNA extraction procedure and the electrophoretic system that was used.

2. Material and methods

2.1. Extraction of RNA

The resected tissues used in this study were provided by Yale-New Haven Hospital, the Mayo Clinic, the Cleveland Clinic Foundation and the Massachusetts General Hospital. After surgery, the tissue was washed in a saline solution and then quick-frozen in liquid nitrogen, generally within 20 min after resection, and stored at −70°C. RNA was extracted with acid phenol [6] or acid guanidinium thiocyanate (abbreviated as GITC, [7]). For acid phenol extraction, the tissue was minced and added to 0.14 M sodium acetate (pH 4.5; 2 ml/g tissue) and a mixture of phenol and chloroform (9:1, v/v; 2 ml/g) in which the phenol had been equilibrated with 0.14 M sodium acetate (pH 4.5). The minced tissue was homogenized. The homogenate was centrifuged in a table-top centrifuge for 10 min at 5,000 × *g*. The aqueous phase was removed and the organic phase was re-extracted with 2 ml/g tissue of 0.14 M sodium acetate (pH 4.5) and centrifuged as above. The two aqueous phases were combined and extracted twice with one volume of phenol and chloroform and once with one volume of chloroform and centrifuged as above. The RNA was precipitated by adjusting the concentration of sodium acetate to 0.3 M and the addition of 2.5 volumes of ethanol.

2.2. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described by Schumacher et al. [5]. The gels were 1 and 2 mm thick for analytical and preparative purposes, respectively. The ratio of acrylamide-to-bisacrylamide was 19:1 (w/w) and the concentration of acrylamide was 10%. The concentration of urea in the second dimension was 7 M. For analytical gels, 50–150 μg RNA were loaded per gel and for preparative gels about 500 μg were used. In the first dimension, xylene cyanol was allowed to migrate about 13 cm and in the second dimension about 30 cm.

The RNA on analytical gels after electrophoresis in the second dimension was stained with silver by first soaking a gel in 50% ethanol and 10% acetic acid overnight, then in 10% ethanol and 1% acetic acid for 1 h. The gel was then stained for 1 h with a 12 mM solution of silver nitrate in water and rinsed with water for 30–60 s. The gel was developed by incubation in a solution of 0.25% formaldehyde in 0.25 M KOH for 10–15 min. Finally the gel was soaked in 60 mM Na_2CO_3 for 15–20 min. The gel was then wrapped in Saran Wrap and photographed.

Preparative gels were stained with ethidium bromide by soaking in 1 μg/ml ethidium bromide in 90 mM Tris, 2.5 mM sodium borate, 1 mM disodium-EDTA, pH 8.3. The RNA was then visualized under UV light. Off-diagonal bands were cut out from the gel and the gel slices were crushed and soaked in 10 mM Tris-HCl, pH 8, 1 mM disodium EDTA, 0.01% SDS, 0.1 M NaCl at 37°C overnight. The RNA was precipitated by ethanol in the presence of oyster glycogen (20 μg/ml; Boehringer-Ingelheim) and stored as a dry pellet at −20°C.

2.3. Enzymatic sequencing of RNA

Small portions of RNA eluted from gels were labeled at either the 3′ or the 5′ end. The labeling was performed with and without prior treatment with alkaline phosphatase to determine whether either end

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was blocked by a phosphate group. The labeled RNA was purified on a denaturing 5% or 8% polyacrylamide gel and eluted as above. Enzymes, with the exception of RNase T1 (CalBiochem), reagents and procedures for sequencing were from Pharmacia. The amount of RNase used was titrated for each individual RNA The reaction mixtures after sequencing reactions were fractionated on denaturing 8% or 12% polyacrylamide gels. Partial alkaline hydrolysis was performed in 50 μl of 50 mM Na_2CO_3 (pH 9.2) and 1 mM EDTA with incubation at 95°C for 5 min. The RNA was then precipitated with ethanol in the presence of oyster glycogen (20 μg/ml)

2 4. Northern blots

RNA (10 μg) extracted with guanidinium thiocyanate [7] was precipitated and dissolved in 15 μl of 1 × agarose gel loading buffer or 15 μl of urea loading buffer (8 M urea, 20 mM EDTA, 0.01% xylene cyanol, 0.01% bromphenol blue) for electrophoresis on a native or a denaturing gel, respectively. The RNA was electrotransferred to a nylon membrane (Amersham, Hybond-N) in 10 mM Tris-acetate (pH 7 8), 5 mM sodium acetate, 1 mM EDTA with a Trans-Blot Cell (Bio-Rad) operated at 250 mA overnight. The RNA was cross-linked to the filter with a Stratalinker (Stratagene). Prehybridization and hybridization were performed with Rapid-Hyb (Amersham) in accordance with the directions from the manufacturer. The oligonucleotide probe AGGAGGAGGACG-GACGGACGGACG, complementary to the 5′ end of RNA X (see Fig 3), was labeled with ^{32}P at its 5′ terminus. Filters were washed first in 1 × SSC and 0.1% SDS, then in 0.2 × SSC and 0.1% SDS, then they were wrapped in Saran Wrap TM and autoradiographed.

3. Results

RNA was isolated from intestinal tissue of patients with active Crohn's disease (CD), ulcerative colitis (UC), non-CD UC inflammatory bowel diseases and adenocarcinoma (distal from visibly affected tissue) by acid phenol or guanidinium thiocyanate extraction and then subjected to two-dimensional gel electrophoresis. The first dimension was run under native conditions and the second dimension under denaturing (7 M urea) conditions. RNA was extracted from nine patients with CD, two with UC, two with diverticulitis and four with adenocarcinoma and fractionated on analytical two-dimension gels (see section 2). Among the nine CD samples, we identified three different off-diagonal species of RNA (Fig. 1). Two of the off-diagonal species (X and Y) were located in the region between 5S (120 nucleotides) and 5.8S (157 nucleotides) rRNA. In the first dimension they migrated together with 5S rRNA and in the second dimension they migrated with 5.8S rRNA (Fig. 1). One of these RNAs (X) was only detected in two out of the nine CD samples and in none of the other samples analyzed on analytical gels. The other off-diagonal RNA (Y) in the region between 5S and 5.8S rRNA was seen in all cases of CD and in lesser amounts in one case of adenocarcinoma but not in any of the other cases. The third off-diagonal species of RNA (Z) migrated in the vicinity of 7S RNA (~300 nucleotides). This RNA was found in only two out of the nine CD samples; one CD and one UC sample also yield a very faint band in the same position as RNA Z (Table 1). RNAs X and Z were not seen in the same CD patients. (Under somewhat different electrophoretic conditions, J.-P. Perreault and S. Altman (unpublished eperiments) observed RNA Z in many samples from patients with CD and UC but not in controls.)

The two RNA-extraction methods yield populations that differed from one another when examined by our methods: GITC (see section 2) yields total RNA and acid phenol yields predominantly low molecular weight RNA which, however, varies in terms of the size distribution in different preparations: sometimes very little RNA larger than 5.8S rRNA is recovered and at other times a small quantity of 28S rRNA is found in the preparation. The only major difference between the two methods is in the amount of the larger RNA (> 5.8S) recovered. By loading more of the GITC-extracted RNA, the same pattern is observed in a two-dimensional gel, with one exception: GITC-extracted RNA yields more 5.8S rRNA and, therefore, species Y, the 5.8S-like rRNA in CD tissue is more easily detected (data not shown).

Results using two different ratios of acrylamide-to-bisacrylamide (19:1 and 40:1) in the gel system (40:1 was employed by Pechan et al. [4]) were compared. More off-diagonal species were detected with the ratio of 40:1 than with the ratio of 19:1, but off-diagonal bands in extracts of control tissue when the ratio was 40:1 (data not shown) were also seen. Therefore a ratio of acrylamide-to-bisacrylamide of 19:1 was employed to facilitate identification of CD-specific species.

RNA X was isolated from a preparative two-dimensional gel and the nature of its end groups was determined by labelling the 3′ and 5′ ends before and after de-phosphorylation (see section 2). The RNA could not be labeled at its 3′ end unless it was first dephosphorylated. The 5′ end could be labeled even without treatment with alkaline phosphatase (data not shown). Thus, the 3′ but not the 5′ end was protected by a phosphate group. Since the RNA could be labeled at its 5′ end without any pretreatment, such as mild digestion with RNase T1, we concluded that the RNA was not circular and, hence, not a viroid. Alternatively, at least part of a population of putative circular molecules has already been nicked. This RNA was treated with either an extract of HeLa cells (S100) under de-branching conditions [8] or with purified de-branching enzyme from yeast (a gift from J. Boeke, Johns Hopkins University; [9]). No changes were observed in its electrophoretic migration properties, an indication that the RNA did not contain a 2′–5′ linkage (data not shown). Enzymatic sequencing of the labeled RNA failed because of the resistance of the RNA to complete digestion by enzymes at concentrations 10- to 100-fold higher then normally used for this purpose. Accordingly, a chemical method of degrading the RNA was chosen in order to generate large fragments, which would presumably have a less hydrogen-bonded structure, and such fragments would be expected to be more susceptible to enzymatic cleavage.

Partial hydrolysis of RNA X with alkali (but not with aniline; [10]) yielded a major product of about half the size of the full-length RNA). This fragment was purified from preparations of RNA X labeled at either terminus and sequenced enzymatically. The 3′ end-labeled fragment yielded a sequence of 33 nucleotides, 5′-CGGCGGCGGCGGCGGUGGCGGCGGCG-

Table 1
Detection of RNA species X, Y and Z in RNA extracted from different tissues by visual inspection of silver-stained two-dimensional gels (see section 2)

Tissue source	Cases	RNA species		
		X	Y	Z
Crohn's	9	1	9*	2
Ulcerative colitis	2	1	0	0
Diverticulitis	1	0	0	0
Adenocarcinoma	4	0	1*	0

*Faint band visible in one case.

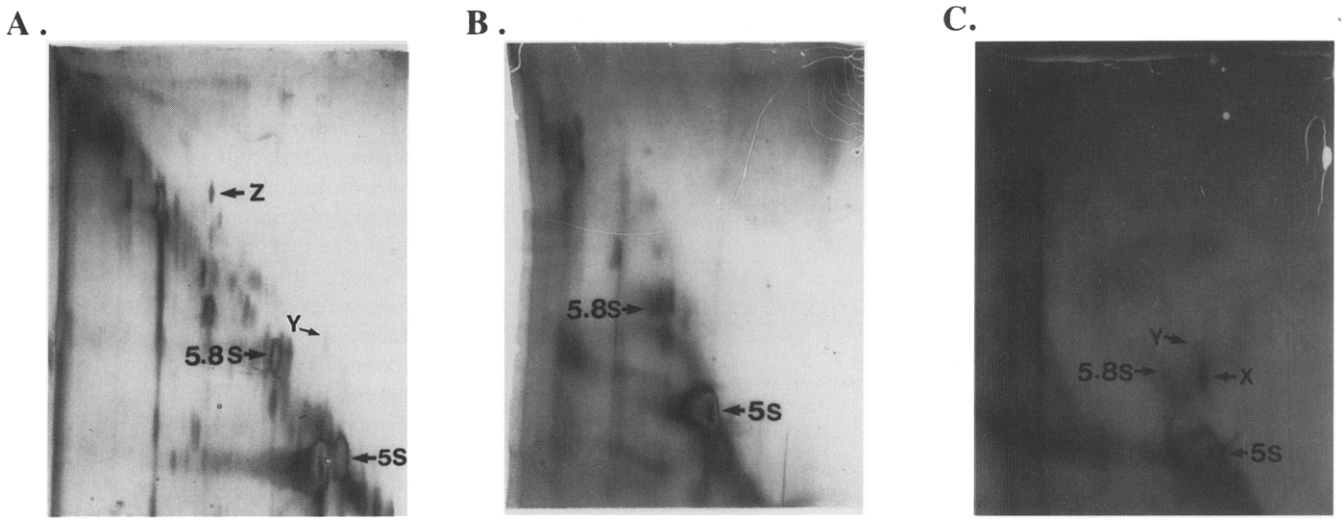

Fig. 1. Two-dimensional gel electrophoresis of RNAs extracted from tissues with acid phenol [6]. The gels have been silver-stained (see section 2) and the positions of 5S, 5.8S and 7S RNA and the off-diagonal spots are indicated by arrows. (A) RNA (60 μg) from CD tissue that contained RNAs Y and Z. (B) RNA (80 μg) from control tissue (resected away from visibly affected area of villous adenoma). (C) RNA (40 μg) from CD tissue that contained RNA, X and Y.

GCGG-3′, starting about 7 nucleotides from the 3′ end (data not shown). The 5′ labeled fragment yielded a sequence of 35 nucleotides, 5′-CCGUCCGUCCGUCCGUCCUCCUCCUCC-CCCGUCUCCG-3′, which started about 4 nucleotides from the 5′ end. A homology search was carried out using BLAST [11] and both sequences were found to be 100% identical to segments of human 28S rRNA ([12]. The two sequences are located 45 nucleotides apart from each other (Fig. 2) and appear to be parts of a rRNA molecule of ~126 nucleotides. This conclusion fits well with the size of RNA X, as determined by two-dimensional electrophoresis (Fig. 1A). This RNA is extremely G-C-rich (88%) and can form a long stem structure that is difficult to denature, thereby accounting for problems in sequencing and its unusual electrophoretic properties. The RNases used for sequencing only cleave single-stranded RNA. The major site of alkaline hydrolysis seems however, to be located near the loop that divides the RNA into two parts of equal size.

For Northern blotting experiments, we designed an oligonucleotide complementary to the 5′ region of RNA X. This oligonucleotide was used to probe a Northern blot of total RNA extracted from tissues from patients with CD, UC, diverticulosis and adenocarcinoma to determine, in part, whether the reason we detected RNA X in only two CD patients (Table 1), and not in the other cases, was an artifact on the silver-stained, analytical two-dimensional gels. The RNA was analyzed on native and denaturing one-dimensional gels under the same conditions as used in the two-dimensional gel system, with the exception that the denaturing gels were run for a shorter period of time (XC migrated about 10 cm).

The strength of signals in the hybridization experiment in the region that corresponded to the migration of RNA X was apparently not well correlated with the amounts of RNA X determined from preparative two-dimensional gels. For example, the sample analyzed in lane 5 in Fig. 3 gave a strong signal on the Northern blot (Fig. 3) but did not yield a significant amount of RNA X on a two-dimensional gel (data not shown). Additionally, one of the controls (lane 1) in Fig. 3 appears to be positive. However, the isolated data from such blotting experiments are not quantitatively reliable. Therefore, membrane filters were scanned with a phosphorimager and the values in each lane were normalized to the amount of 5S rRNA in that lane, as determined from re-analysis of the same filters with an appropriate probe. The normalized values are shown in Fig. 3 and indicate that, on average, these values are higher in the case of CD samples than in the case of the controls or UC (The

Table 2
Ratios of the level of RNA X to 5S RNA in different tissues

Expt. no.	Extraction method	Controls	Samples	CD	Samples	UC	Samples	Others	Samples
1	GITC	1.21	3	2.04	9	0.69	2	–	
2	GITC	1.19	7	1.21	5	0.92	3	–	
3	GITC	1.26	6	3.69	10		–	1.36	2
4	GITC	1.08	2	1.22	4	1.58	2	0.93	4
5	acid phenol	1.62	9	1.24	7	–	–	–	–
Averages*		1.21	(18)	2.25	(28)	1.04	(7)	1.17	(6)

*Excluding Expt. 5.
RNA was probed on Northern blots with either a probe specific for that of 28S rRNA (CTAACCAGGCCCGACCCTGCTTAGC) or a probe specific for 5S rRNA. Blots were analyzed with a phosphorimager (Fuji). Ratios were calculated for each lane (tissue sample) on each filter and averages were calculated for all the cases analyzed on each filter.

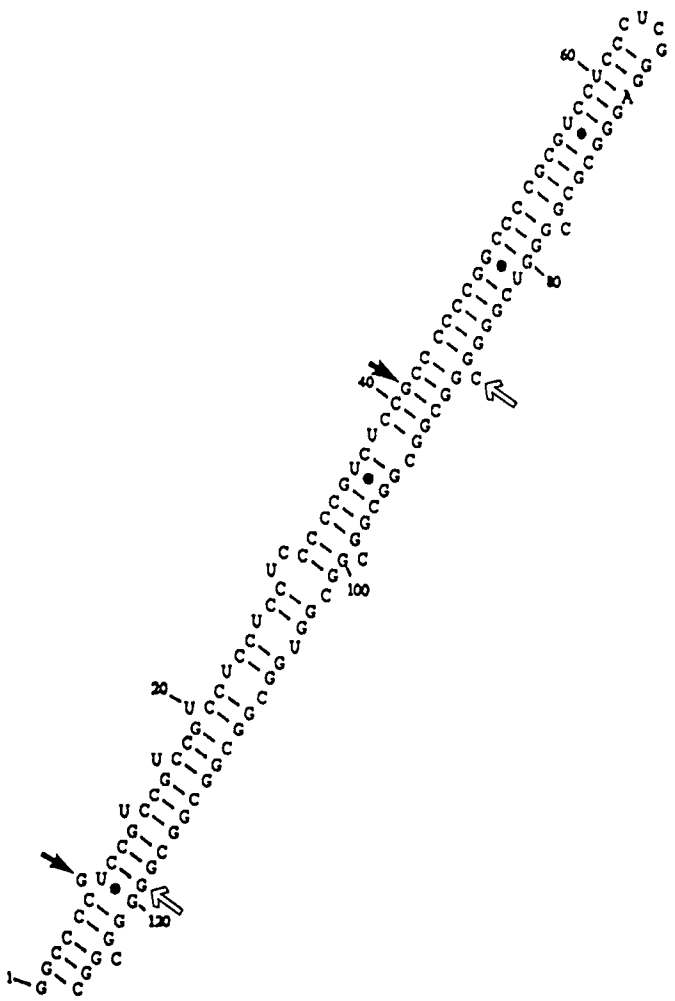

Fig. 2. The sequence and proposed secondary structure of human 28S rRNA, variable region 2 stem 9 (V2–9), from Gorski et al. [17]. The sequence between the black arrows is identical to that determined for 5′ labeled RNA X and the sequence between the open arrows is identical to that determined for 3′ labeled RNA X.

diagnosis of CD in the patient whose RNA was analyzed in lane 4 was uncertain.)

Several Northern blotting experiments similar to the one shown in Fig. 3 were performed to determine whether any conclusions could be drawn with respect to the amount of the signal that corresponded to RNA X at the expected position in the gels and the pathology of the tissue from which the RNA was extracted. Adenocarcinoma and non-malignant tissue were included as controls in these experiments. The results showed (Table 2) that, in individual experiments, total RNA extracted with GITC from CD tissue contained, on average, more of RNA X than the RNA extracted from control tissue. However, because there was considerable variation in the absolute numbers (two-fold or more within each group) obtained in these experiments, deviations from the mean values for the control and the CD tissues overlapped. When results from all the experiments were considered together, a similar conclusion was reached to that reached after individual experiments.

A comparison of the data for GITC-extracted RNA with data for RNA extracted with acid phenol (Table 2) confirmed that the yield of RNA X was diminished when the latter method was used. Indeed, with acid phenol, the average levels of RNA X in control tissue appeared higher than those in CD tissue. Our results indicate that Northern blot experiments to detect the presence of RNA X cannot be used for diagnostic purposes in individual cases. Independent evidence of the presence of RNA X obtained from visual inspection of stained two-dimensional gels must be used to confirm the presence of this species of RNA.

Northern blots were also prepared with RNA that had been extracted with phenol from S30 preparations from which membrane fragments and bound RNA and ribosomes had been removed. In RNA from both CD and control tissues, a distinct band in the expected location of RNA X was observed (data not shown) suggesting that all tissues had active nuclease(s) that could specifically cleave 28S rRNA. This observation is consistent with that of Houge et al. [13]. Furthermore, the recovery of RNA X from S30 preparations was not as complete as that from GITC extracts of total RNA from whole cells. Attempts to detect RNA X by PCR techniques were unsuccessful. Accordingly, the utility of RNA X as a diagnostic tool for CD is presently limited unless large amounts of material for two-dimensional gel electrophoresis are available after resection.

RNA Y was isolated from two patients with CD and labeled at its 3′ end after de-phosphorylation. Analysis of the labelled RNA yielded the sequence UGUGAAUUNCAGGACACA-NUGAUCAUCGACACUUCGAA-3′ (N = A, G, C or U). This RNA was identified as 5.8S rRNA by a BLAST homology search of database [11]. The sequence that matched 5.8S rRNA was the same distance from the 3′ end of RNA Y as the homologous sequence in 5.8S rRNA is from the 3′ end of the latter molecule. RNA Y was found in all nine cases of CD on two-dimensional gels and not in any cases of UC or non-CD-UC inflammatory bowel disease (Table 1). However, a faint spot was seen at the same position on two-dimensional gel analysis of one of the controls. The identity of the sequence was confirmed in RNA extracted from a total of three patients with CD. Species Z was also found on two of the latter gels (see below). We do not know if RNA Y is a 5.8S species of rRNA that is expressed uniquely in CD tissue or whether it is a novel conformer of 5.8S rRNA. In either case, it is a possible diagnostic marker for CD, if sufficient amounts of tissue are available for analysis.

RNA Z migrated as an RNA in the same size range (~300 nucleotides) as but closer to the diagonal than the off-diagonal RNA detected by Pechan et al. [4]. The difference could be due to the difference in the ratio of acrylamide to bisacrylamide used in our experiments. This RNA was labeled at its 5′ end without prior pre-treatment but was protected by a phosphate group at its 3′ end. No hydrolysis of this RNA in enzymatic RNA-sequencing reactions was observed unless the amounts of RNases used were much higher than those used for RNA X and RNA Y (~10 U compared to ~1 U for the 28S rRNA fragment). Even with very high levels of RNases we were only able to obtain a short, partial sequence: 5′-CGGYGGYGGGYUN-ANNGUG-3′ (Y = C or U; N = A, G, C or U). This sequence resembles but is not identical to the 3′ portion of RNA X. Many RNAs in human cells are derived from DNAs with GGY repeats. The amount of RNA fragments of the size of RNA Z was higher in diseased tissue than in the controls.

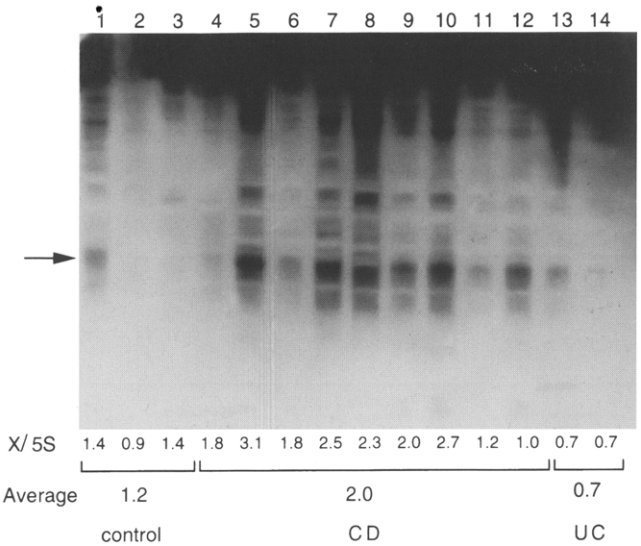

Fig. 3. Northern blot analysis of total RNA that was extracted with GITC from control, CD and UC tissues. RNA (10 μg) was fractionated on a 10% polyacrylamide gel, transferred to a Hybond-N membrane and probed with an oligonucleotide complementary to the 5′ region of V2–9 (Fig. 3). Lanes 1–3 = controls; lanes = 4–12, CD tissues. Lane 13 and 14 = UC. The arrows indicate the position of V2–9 RNA that was identified as a positive control in the CD sample shown in lane 4.

4. Discussion

We examined RNA in tissues from patients with Crohn's disease to determine whether any of this RNA was similar to viroid RNA, a possibility raised earlier by Pechan et al. [4]. RNA extracted from 9 CD, 2 UC, 1 diverticulitis and 4 control tissues was analyzed by two-dimensional gel electrophoresis and three different off-diagonal RNA species, found in isolates from CD tissues were studied. Two of these RNAs (X and Z) were only seen in two cases each. RNA X (~126 nucleotides) was identified as a fragment of 28S rRNA. RNA Z, a mixture of larger fragments, yielded very limited sequence information. The third off-diagonal RNA (Y) was observed in all CD tissues and was identified as a conformational or sequence variant of 5.8S RNA. All of these RNAs were GC-rich and were probably only partially denatured in the denaturing dimension of the two-dimensional gel system. This feature might explain their unusual mobility in this system.

One explanation for the off-diagonal RNAs seen in CD tissue and not in control tissue is that off-diagonal RNAs are products of the breakdown of normal cellular RNA that results from inflammation in affected CD tissue. We treated purified ribosomes with cell extracts from CD and from control tissue and we saw the same pattern of cleavage of rRNA in both cases. The RNases responsible for the degradation of RNA in affected CD tissue may be present in both CD and control tissue (Fig. 3), albeit not in equal amounts.

The presence of RNA X, a degradation product of 28S rRNA, could be the result of a secondary effect of an infectious process that stimulates production of interferon, which, in turn, via 2′-5′A, activates RNase L [14]. After addition of 2′-5′A to crude extracts of CD tissue under conditions that might be expected to activate RNase L, we observed no differences in the pattern or extent of the specific cleavages of rRNA that we reported here (data not shown).

Cleavage of 28S rRNA has also been observed in rat myeloid leukemia cells that have been stimulated to undergo apoptosis [13,15]. We searched for the specific fragmentation of DNA, a marker for apoptosis, in CD tissue but found none (data not shown). Furthermore, there is no significant increase in numbers of apoptotic cells in CD tissue as compared to control tissue [16]. The pattern of RNA degradation in necrotic tissue [13] is generally different from both the specific cleavages observed in apoptotic tissue and from those that we report here. These results indicate that the cleavages observed are very likely the result of an ordered process that is more active in the tissue of patients with CD than in control tissue. Such cleavages may be the result of activation of resident nucleases or of activation of helicases or other factors that render these RNAs more susceptible to cleavage in the diseased tissue. There must be many pathways that can lead to specific cleavage of rRNA as a prelude to cell injury or death.

RNA Y, seen selectively in CD tissue, seems not to be a breakdown product of a larger RNA. Its electrophoretic properties, namely, its faster migration in the first dimension, are an indication of a more compact structure rather than of an RNA species that is shorter in nominal length than 5.8 S RNA. In CD tissues, conformational changes as a result of alterations in levels of RNA helicase or chaperonin, as well as possible minor sequence differences in the 5.8S rRNA can be the cause of the anomalous electrophoretic mobility.

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