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Characterisation of messenger RNA extracted post-mortem from the brains of schizophrenic, depressed and control subjects

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SUMMARY Messenger RNA, obtained from post-mortem brain of 10 schizophrenics, five depressed patients and 10 control subjects, was characterised with respect to a number of parameters. It was found that post-mortem delay was not the major factor in determining RNA yield, size (as determined by cDNA synthesis) and biological activity. Biological activity, as determined by in vitro translation in a reticulocyte-lysate system, could be observed using messenger RNA from periods of 0 to 84 hours post-mortem. Two-dimensional gel analysis of the newly-synthesised radiolabelled products obtained from this material revealed several hundred individual species but no consistent degradation of any particular species with post-mortem delay. It is suggested, therefore, that pre-mortem changes are as important as post-mortem changes in determining RNA yield, size and biological activity. Although no consistent difference could be found between patients and controls using any of these parameters, this study confirms that, by isolating messenger RNA from post-mortem human brain, valuable information can be gained on gene expression in psychiatric disorders.

There is considerable evidence from classical family, twin and adoption studies for a genetic predisposition to many types of mental disorder, including schizophrenia and affective disorder.[1 2] The involvement of specific genetic elements, and therefore transcriptional mechanisms, in the aetiology of affective disorder has recently been confirmed using molecular genetic techniques, with the reported linkage of affective disorder to the insulin/Ha-ras-1 locus on chromosome 11 in a large Amish pedigree.[3] Despite this finding, the existence of genetic heterogeneity[4 5] and uncertainty about the true values of penetrance for the predisposing genes confound the ability of DNA linkage methodology to define the genetic lesions further. It is therefore important to identify transcribed genes which can be envisaged to be associated with those disorders in order to supplement genetic data. One such approach is to compare brain poly(A)-containing messenger RNA populations from normal and psychiatrically ill patients. With this in mind, we have studied the effects of post-mortem delay on the biological activity and size of poly(A)-containing messenger RNA isolated from schizophrenic, depressed and control brain tissue. Some of the results shown here have already been presented in a preliminary communication.[6]

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Methods

Brain samples

Brain tissue (10 schizophrenics, five depressed patients and 10 control subjects) was obtained from Dr RH Perry (Newcastle-upon-Tyne General Hospital) and Dr G Reynolds (MRC Neurochemical Pharmacology Unit, Cambridge). Material from both the frontal cortex and caudate nucleus was used. Subjects had died from a variety of causes and post-mortem delay (the time between death and tissue dissection/freezing) varied from 3 to 84 hours. In addition, control tissue (no post-mortem delay) was obtained during a temporal lobe biopsy performed by Mr CE Polkey (Neurosurgical Unit, Maudsley Hospital).

RNA extraction

Total cellular RNA was extracted from frozen brains essentially as described previously,[7] using guanidinium thiocyanate and a caesium chloride centrifugation step.

Poly(A)-containing messenger RNA was obtained from total cellular RNA by using oligo (dT)-cellulose chromatography.[8] Both total cellular RNA and poly(A)-containing messenger RNA were estimated by A260 measurements.[8]

Protein synthesis and analysis

Poly(A)-containing messenger RNA (20 $\mu g\ ml^{-1}$) was used to direct the synthesis in vitro of proteins in the reticulocyte-lysate system, using [^{35}S] methionine as radiolabelled precursor, as described previously.[7] Translation products were analysed using high-resolution two-dimensional gel electrophoresis as described by O'Farrell,[9] for isoelectric focusing (ISODALT system), and O'Farrell *et al*,[10] for non-equilibrium pH gradient electrophoresis (BASODALT system). Minor modifications were employed in sample solubilisation and pH gradient generation.[7]

Spot intensities on fluorograms were measured by the densitometric scanning technique of Quitschke and Schechter,[11] where a value proportional to the volume integral is determined. Intensities were normalised by a comparison with two standard spots (see figs 4 and 5) whose intensity did not vary significantly from controls (as measured by Student *t* test values; data not shown).

cDNA synthesis and analysis

cDNA was synthesised by a modification of the procedure described by Arrand.[12] The incubation mixture (10 μl total volume) contained: 75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH 7·5), 20 mM dithiothreitol, $2{\cdot}23 \times 10^{-3}$ A260 units oligo $(dT)_{12-18}$, 12U placental RNAsin, 100 nM each of dATP, dCTP, dGTP, dTTP, [α-^{32}P]dCTP (0·1 μCi; 420 Ci $mmol^{-1}$), 50 $\mu g ml^{-1}$ actinomycin D, 200U murine moloney leukaemia virus reverse transcriptase and 50 ng poly(A)-containing messenger RNA. After incubation (37°C, 1 h) the cDNA was ethanol-precipitated and washed three times in 75% v/v ethanol: 25% v/v 0·1 M sodium acetate. The cDNA was analysed for size in. a denaturing agarose gel (1% w/v agarose in 30 mM NaOH, 50 mM NaCl, 1 mMEDTA), using alkaline electrophoresis buffer (30 mM NaOH 1mMEDTA).

Results

RNA recovery

Recovery of both total cellular RNA and poly(A)-containing messenger RNA was variable among different brain samples but there were no significant differences between the groups analysed, as measured by Student *t* test values (tables 1 and 2). There was no marked decrease in total cellular RNA with post-mortem delay (fig 1) and, in addition, age at time of death did not affect the RNA yield (results not shown). Similarly, there was no significant change in the poly(A)-containing messenger RNA (expressed either as a percentage of total RNA or in terms of $\mu g\ g^{-1}$ brain) either with post-mortem delay or with age over the range studied (results not shown).

Messenger RNA size

Poly(A)-containing messenger RNA size was estimated by reverse transcription into cDNA followed by cDNA sizing (fig 2). Although this underestimates messenger RNA size owing to premature termination by reverse transcriptase, relative size distributions of samples can be observed. cDNA sizes showed continuous distributions in the range 200–1400 nucleotide residues. There was no significant change in size with post-mortem delay over the time period considered.

Table 1 *Comparison of total cellular RNA of brain samples*

Group	*Number of samples*	*Total cellular RNA (μg/g (SD))*	*Age (years (SD))*	*Post-mortem delay (hours (SD))*
Controls*	6	167 (58)	73 (6·9)	36·5 (27·7)
Schizophrenics*	8	184 (74)	72 (6·8)	42·2 (26·1)
Depressives*	5	207 (89)	76 (4·5)	29·6 (10·1)
Total†	30	174 (60)		

*Comparison of cortex values only.
†Including cortex and caudate values plus fresh biopsy material.

Table 2 *Comparison of poly(A)-containing messenger RNA of brain samples*

Group	*Number of samples*	*Poly(A)-containing mRNA (% of total cellular RNA (SD))‡*	*Age (years (SD))*	*Post-mortem delay (hours (SD))*
Controls*	5	3·93 (1·63)	73 (7·5)	43 (25·4)
Schizophrenics*	5	2·75 (1·72)	70 (7·4)	58·5 (17·4)
Depressives*	5	2·79 (1·76)	76 (4·5)	29·6 (10·1)
Total†	16	3·19 (1·62)		

*Comparison of cortex values only.
†Including fresh biopsy material.
‡A similar analysis yielded no significant differences when expressed in terms of μg mRNA/g brain tissue.

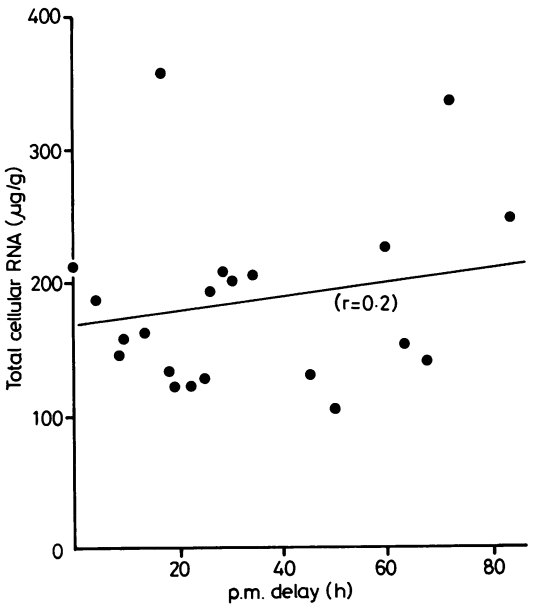

Fig 1 *Change in total RNA yield with post-mortem delay in tissue sampling. RNA was isolated and extracted as described in the Methods section. The number in brackets denotes the correlation coefficient value.*

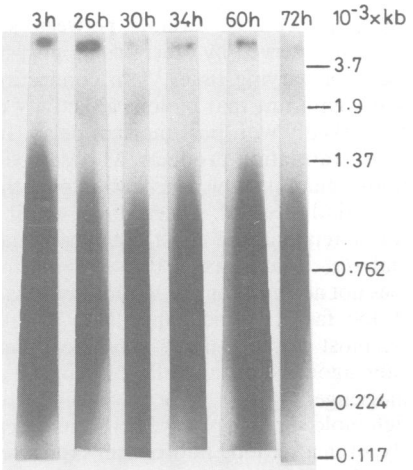

Fig 2 *cDNA size estimation. cDNA was synthesised from poly(A)-containing messenger RNA and analysed as described in the Methods section. Key: numbers above lanes represent post-mortem delay times; right-hand scale represents the migration of Mr markers run on the same gel.*

Biological activity of messenger RNA

All samples analysed showed biological activity in the in vitro translation system, although there was considerable variation between samples (table 3). Student *t* test results indicated that there were no significant differences between groups (table 3) and, perhaps surprisingly, there was no marked reduction in biological activity with post-mortem delay (fig 3).

Translation products were analysed using both equilibrium (fig 4) and non-equilibrium (fig 5) two-dimensional gel electrophoresis. Analysis of translation product fluorograms revealed a number of proteins which could be consistently identified and over 200 individual radiolabelled species could be detected. No significant amounts of high molecular weight species (Mr greater than 90000) were present in any of the samples analysed. However, there was no consistent shift to lower molecular weight species with increased post-mortem delay, nor any consistent loss of specific individual species (results not shown). It was apparent, therefore, that molecular weight distribution and intensity of specific species was not a simple function of post-mortem factors.

Comparisons of translation product patterns between normal, schizophrenic and depressed patients revealed some differences in relative intensities of specific proteins on some gels (figs 4 and 5). These differences are quantified in table 4. However, comparisons of translation product fluorograms from all samples indicated that there were no consistent differences between either normal and schizophrenic or normal and depressed groups.

Discussion

RNA recovery

In this study, the yield of both total cellular RNA and of poly(A)-containing messenger RNA from different samples was variable, although the results were consistent with previously published values.[7 13–16] Such variation for total RNA is probably not due to impurity of the sample, since RNA obtained by the method used here is relatively free from both DNA[14] and protein (different samples had similar A260:A280 ratios; results not shown). Contamination of the sample cannot be discounted for messenger RNA, however, since messenger RNA isolated from an oligo(dT) column contains relatively high levels of ribosomal RNA,[7 14] although these levels are still small compared with the total variability observed here. It may be that factors such as tissue dissection, handling and storage play an important part in determining the quantity of RNA recovered. Inclusion of traces of white matter in the sample would affect relative yield, since RNA levels in white matter are only half those in grey matter.[17]

Table 3 *Comparison of messenger RNA biological activity of brain samples*

Group	Number of samples	Biological activity‡	Age (years (SD))	Post-mortem delay (hours (SD))
Controls*	5	3·94 (2·42)	73 (7·5)	43 (25·4)
Schizophrenics*	5	4·18 (2·64)	70 (7·4)	58·5 (17·4)
Depressives*	5	3·32 (1·10)	76 (4·5)	29·6 (10·1)
Total†	18	3·98 (2·21)		

*Comparison of cortex values only.
†Including cortex and caudate values plus fresh biopsy material.
‡As measured by trichloracetic acid precipitation of synthesised proteins (7); values quoted are -fold stimulation over the blank value and (SD).

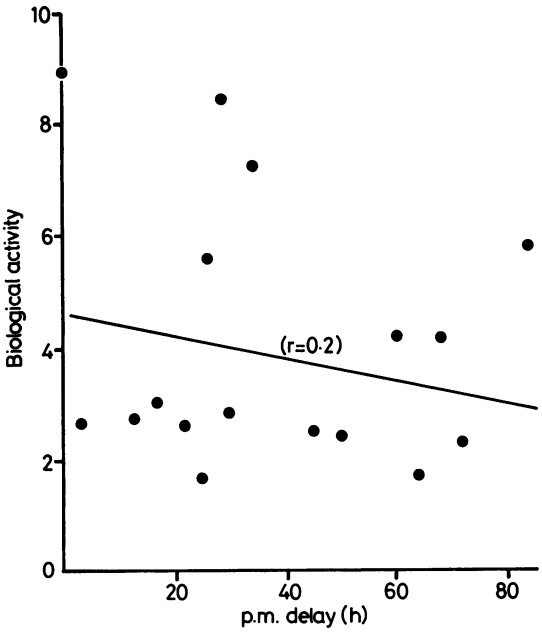

Fig 3 *Change in biological activity of messenger RNA with post-mortem delay in tissue sampling. Biological activity was measured by trichloroacetic acid precipitation of synthesised proteins.*[7] *Values quoted are -fold stimulation over the blank. The number in brackets denotes the correlation coefficient value.*

RNA breakdown, both pre- and post-mortem, thus remains the most obvious determinant of yield of both total RNA and poly(A)-containing messenger RNA. However, there was no correlation observed between low RNA yield and either size of messenger RNA (as judged by reverse transcription) or size of translation products (see below).

Effects of post-mortem delay

From the results obtained here, and in agreement with others,[13-15] it appears that post-mortem delay in tissue sampling per se does not significantly affect RNA recovery. Moreover, and surprisingly, post-mortem delay did not appear significantly to affect messenger RNA size, as judged by cDNA analysis. Rather, it is possible that pre-mortem changes may play a more important role than those post-mortem. In support of this proposal, Perry *et al*[18] have shown that, when death follows a prolonged period of severe illness, significant differences can be observed in brain tissue pH, as well as some brain amino acid and enzyme levels, compared with those values in patients where death either occurred suddenly or after a period of normal health. Variability in the recovery of total cellular RNA in this study may therefore be a reflection of individual variations in agonal state.

Biological activity

High-resolution two-dimensional gel electrophoresis has been used in a number of studies examining changes in *in vitro* translation products using messenger RNA isolated from both normal and pathological human brain.[7 14 19-22] As with RNA yield, recovery of biological activity (determined by trichloroacetic acid precipitation) was variable between samples. This has been observed by others using post-mortem brain tissue of varying times.[14] In connection with this it was significant that biological activity did not decrease markedly with post-mortem delay. In addition, size of translation products, as observed on the fluorograms, did not show degradation over the post-mortem period studied. Therefore, whilst RNA degradation remains the most significant factor in such analyses, it appears that post-mortem delay alone does not account for this variability. It is probable that the factors which appear to affect RNA recovery, most notably tissue dissection, handling, storage and agonal state, also affect biological activity of the messenger RNA.

No high molecular weight species were observed in this study, even after long fluorogram exposure times. However, this did not appear to be due to post-mortem breakdown of messenger RNA, since messenger RNA derived from freshly frozen biopsy material produced translation products of similar size distribution. Differences in translation product sizes observed here, when compared with earlier results,[7]

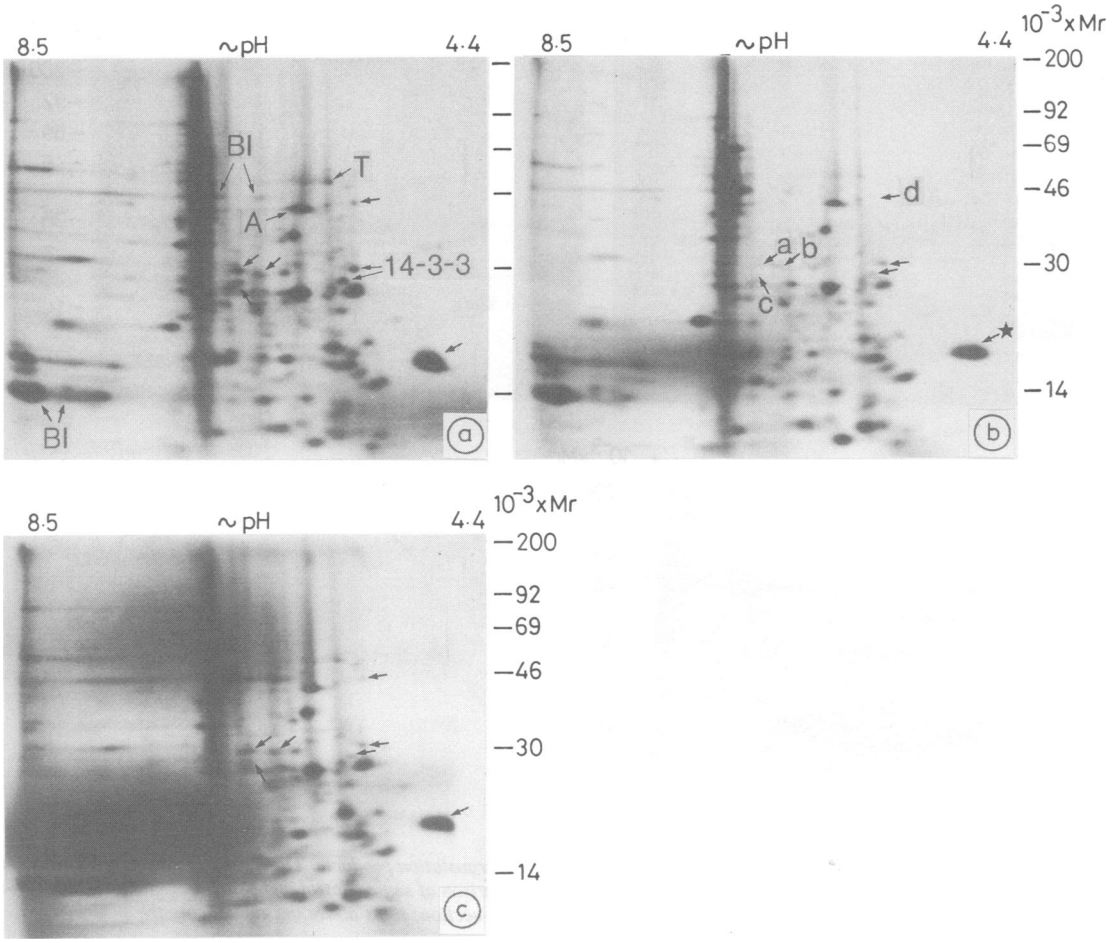

Fig 4 *Two-dimensional ISODALT polyacrylamide-gel analysis of translation products. In vitro translation and gel analysis were performed as described in the Methods section. The sample contained 6 × 10^4 acid-precipitable c.p.m. Key: (a), translation products of poly(A)-containing messenger RNA isolated from control subjects; (b) translation products of poly(A)-containing messenger RNA isolated from schizophrenics; (c) translation products of poly(A)-containing messenger RNA isolated from depressives; a, b, c, d, spots whose fluorographic intensities changed in a comparison of some control and psychiatric subjects; (★), the standard spot for the ISODALT system, as described in the Methods section; right-hand scale represents the migration of Mr markers run on the same gel; numbers above the gel indicate the pH of the ends of the isoelectric-focusing gels. Abbreviations: A, actin; T, tubulin; 14-3-3, brain 14-3-3 protein; Bl, [^{35}S] methionine-binding proteins of the lysate system.*

may reflect the developmental period studied (adult brain compared with foetal brain), since similar size distributions have been observed by other authors in adult post-mortem brain.[20 22] In addition, no consistent selective loss of individual species was observed with increased post-mortem delay.

The observation that intact messenger RNA, capable of translating proteins in the in vitro system, was obtained at post-mortem delays of up to 84 hours is significant, since such translation is highly dependent on the integrity of the 5[1]-terminal cap structure in brain messenger RNA.[23] This extends the results of other studies indicating that messenger RNA seems intact for periods of up to 36 hours post-mortem[15 21] and confirms that the use of messenger RNA samples, extracted post-mortem from human brain, is likely to

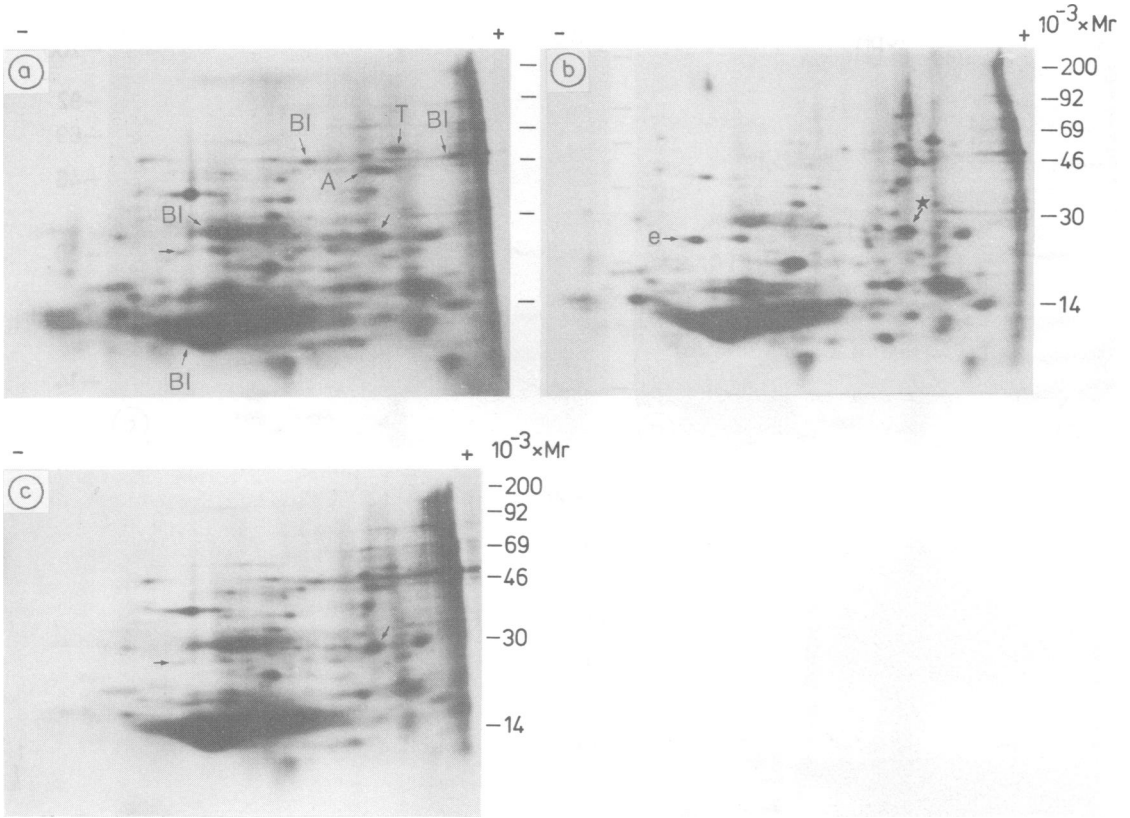

Fig 5 *Two-dimensional BASODALT polyacrylamide-gel analysis of translation products. The legend is generally the same as in fig 4. Key: e, a spot whose fluorographic intensity changed in a comparison of some control and psychiatric subjects; (★), the standard spot for the BASODALT system, as described in the Methods section; signs above the gel indicate the electrode polarity for the BASODALT separation.*

Table 4 *Comparison of specific spot intensities on fluorograms shown in figs 4 and 5*

*Spot**	*Schizophrenics (%)†*	*Depressives (%)†*
a	4	93
b	5	100
c	0	96
d	0	100
e	3333	89
14-3-3 (basic)	35	35
14-3-3 (acidic)	26	26

*Spot designations as in figs 4 and 5.
†Intensities were measured and normalised against the standard spots shown in figs 4 and 5. Values are expressed as a percentage of control intensities.

provide information on expressed genes in neuropsychiatric conditions. Indeed, the data presented in this study suggest that the state of the tissue premortem may be more important than post-mortem delays in tissue sampling in determining both recovery and biological activity of RNA species. It is possible, however, that important changes occur within 33 hours postmortem; we are therefore currently studying samples of this time interval.

With these considerations in mind, in this study we found no consistent significant differences on fluorograms between the control and psychiatric groups which suggests that, at least in the samples studied here, changes in abundant messenger RNA populations are not a feature of the disease state. Fur-

ther analysis of the rarer populations using cDNA cloning techniques is now under way to obtain a more complete picture of these processes.

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