In addition, one can construct a curated library of PSI-BLAST compatible profiles and modify the program in order to compare a query directly to this library. We hope that future refinements that perhaps incorporate some of these ideas will further enhance our ability to make sense of protein sequences.

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Eukaryotic rRNA methylation: the calm before the Sno storm

Much excitement has arisen from the discovery that small nucleolar RNA molecules (snoRNAs) function as guides for post-synthetic modifications of eukaryotic rRNA[1–6]. The title of one review, 'Sno storm in the nucleolus: new roles for myriad small RNPs'[6], abundantly conveys the excitement. A prerequisite for this work was the accurate mapping of the numerous modified nucleosides within eukaryotic rRNA. My colleagues and I had the good fortune to contribute to this earlier work, especially the mapping of the RNA methyl groups. Here, I look back on the mapping work, which, in retrospect, was a mini golden era of 'calm before the Sno storm'.

Albert Einstein College of Medicine, 1967–1969

In January 1967, I arrived as a postdoc in Jim Darnell's laboratory at the Albert Einstein College of Medicine, New York. I had completed my PhD, on ribosome-catalyzed peptidyl transfer, under Robin Monro at the Medical Research Council Laboratory for Molecular Biology, in Cambridge. Robin had been a postdoc with Fritz Lipmann and was an early leader in the characterization of the partial reactions of protein synthesis. The subject had interested me greatly, and my contribution made a mark[7], but I had also become interested in RNA biosynthesis in animal cells through reading Jim Darnell's work; when Jim offered me a postdoctoral position, I accepted enthusiastically.

Jon Warner – who was already well known for discovering polysomes during his PhD with Alex Rich at MIT – was closely associated with Jim's research group. Because of my background in ribosomes, we agreed that I should work jointly with Jon and Jim. This was an ideal arrangement and was further enhanced by the stimulating overall environment. Harry Eagle (of Eagle's medium) had been influential in attracting several leading cell biologists to 'Einstein', and there were excellent interactions between research groups. A wide range of topics was amenable to the (then) illuminating methods of radioactive labelling, cell fractionation, sucrose-gradient centrifugation and polyacrylamide-gel electrophoresis, and many features of cellular and viral composition and biosynthesis were being revealed.

Sheldon Penman had developed a cell-fractionation procedure that allowed purification of the nucleolar precursors of rRNA (Ref. 8). When I arrived, Jon Warner was developing this procedure further for isolation and characterization of nucleolar preribosomal RNP particles[9]. In a separate study, Jim's group discovered 5.8S rRNA (initially called 7S rRNA)[10]. The discovery exemplified Jim's perceptive eye; he had noticed that 28S rRNA sedimented slightly more slowly after extraction with hot phenol than after extraction with cold phenol. He guessed that a small piece of noncovalently attached RNA might be released by heat treatment, and he and colleagues sought and characterized this RNA[10]. Jim and Jon were both most interested in regulation, however. One approach to regulation was to observe the effects of depriving cells of a nutritionally essential amino acid – and thereby slowing down protein synthesis to turnover levels – on ribosome formation. I used valine deprivation as a model[11]; withholding this essential amino acid led to a reversible slowing down of pre-rRNA synthesis and processing, but not to complete cessation.

 PII: S0968-0004(98)01304-8

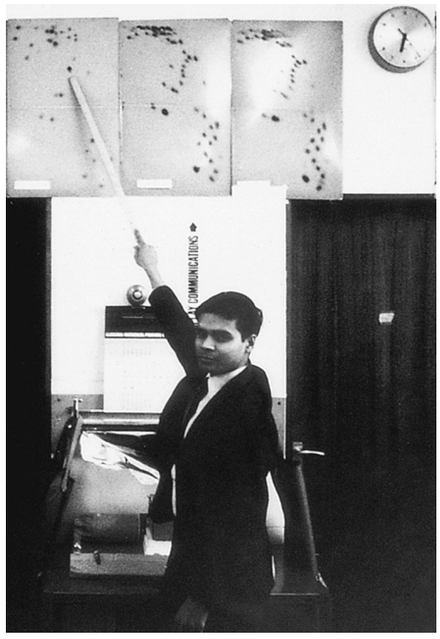

Figure 1
Salim displays his RNA methyl fingerprints in the Glasgow lab.

Mike Vaughan, a postdoc in the lab, had obtained very different results when studying methionine deprivation. He was interested in the observation that eukaryotic rRNA appeared to be more heavily methylated than prokaryotic rRNA, and to exhibit a preponderance of 2′-*O*-methylation and only a minority of base methylation. Greenberg and Penman[12] had shown that (most) rRNA methylation in HeLa cells occurs rapidly on pre-rRNA, apparently on nascent chains. Mike and his colleagues asked whether pre-rRNA methylation is essential for ribosome formation. Exploiting the fact that methionine is the source of methyl groups for nucleic acid methylation, and that it is nutritionally essential in animal cells, they showed that, during methionine deprivation, pre-rRNA methylation is largely suppressed and rRNA maturation is completely inhibited. They inferred that pre-rRNA methylation is essential for ribosome maturation[13].

I mentioned these findings in a short review article[14]. A member of the editorial staff of *Nature* had visited Einstein to commission a review on rRNA and ribosome biosynthesis. Jim and Jon both had other writing commitments, so the offer came to me. It was a great opportunity for a fairly junior scientist, and I worked hard to produce an informative account of the state of the art. There was a small glitch in publication. That summer (1968), I had planned a mountaineering trip to a remote area in northern Canada. The area was accessible only by float plane (sea plane) from Watson Lake on the Alaska Highway. I asked for the proofs to be sent to the Watson Lake post office. Upon emerging from the wilds and collecting the proofs, I found that my short description of the newly discovered 7S rRNA had been embellished with the words 'if it is not an artefact'. I deleted the phrase and mailed the proofs back straightaway, but I was too late – the article had gone to print! It was an embarrassed postdoc who arrived back in the lab after his long holiday. Fortunately, however, the review was a success from all other points of view and, to everyone's pleasure, reprint requests came pouring in.

Glasgow

In the spring of 1969, I took up a lectureship in Biochemistry at the University of Glasgow. Martin Smellie, who had been my PhD external examiner, was a professor there and had encouraged me to apply. The head and founder of the department was Professor J. N. Davidson, whose small book *The Nucleic Acids* I had read many years previously as an undergraduate. At first I was in awe of 'JND', but I soon perceived that he considered me an asset, and he used his influence to offer me two research students during my first year. The department was in the process of installing good cell-culture facilities, through a grant from the Wellcome Trust, so I was able to continue using the cell labelling and fractionation methods I had learned at Einstein.

My first student, Jim Shepherd (who is now Professor of Pathological Biochemistry at Glasgow Royal Infirmary), worked on a peptide-mapping project, studying ribosomal proteins. We were seeking to extend Jon Warner's ribosome-assembly studies[9] by labelling ribosomal proteins with [^{35}S]amino acids and then displaying tryptic peptides in two-dimensional fingerprints. The work met with some early success[15], but the number of labelled peptides was too large for full characterization, and two-dimensional fractionation of intact ribosomal proteins – as developed by Wittmann's group – took over in such work soon afterwards.

Initially, I was reluctant to take another student during my first year, but JND did not accept 'no' for an answer. He assured me that the student, Salim (Fig. 1), who was from Pakistan, was excellent. I therefore accepted JND's offer and began to think out a project that had been at the back of my mind. In the mid-1960s, Fred Sanger and colleagues had developed two-dimensional separation methods for RNA oligonucleotides[16]. Just before I left Cambridge, a new PhD student of Sanger, Peter Fellner, had started to study methylated oligonucleotides from enzymatic digests of *Escherichia coli* rRNA. His work was published[17] shortly before I arrived in Glasgow. It was a small logical step to conceive of applying the methodology to eukaryotic rRNA and pre-rRNA from HeLa cells.

When Salim arrived, we discussed the possibility. I calculated the amounts of cells and labelled methionine, and the labelling times, that would be needed to produce methyl-labelled rRNA and pre-rRNA that had the necessary specific activity for T_1-RNase digestion followed by two-dimensional electrophoresis (fingerprinting). Fingerprinting, however, was new and daunting ground to me, and we did not have the equipment. Fortunately, Bob Williamson, who was then working nearby at the Beatson Institute for Cancer Research, had the necessary expertise and equipment, which he had been using to study 5S rRNA. He offered to collaborate, and our first results showed immediately that we were in business. 18S and 28S rRNA yielded different methyl fingerprints and, as predicted, the 45S fingerprint contained 18S and 28S spots. The results[18] were only a first glimpse of what would eventually unfold, but they were sufficiently promising to justify the installation of a high-voltage-electrophoresis facility in the basement of the Biochemistry Department. They also helped me to obtain independent grant money.

Salim was amply fulfilling JND's assurance that he was an excellent student, and we made an effective pair. Early on, I was interested in establishing the qualitative and quantitative relationships between methylation of rRNA and pre-rRNA, whereas Salim was interested in sequencing the methylated oligonucleotides, using [^{14}C]methyl-labelled and ^{32}P-labelled rRNA separately or in combination, and a variety of enzymatic and chemical degradation procedures.

One problem was to obtain sufficient yields of all of the methyl-labelled nucleolar pre-rRNA species, including the quantitatively minor 41S intermediate, for fingerprinting. Weinberg and Penman had shown[19] that these species accumulated during infection of HeLa cells with poliovirus. I therefore arranged to revisit Einstein and work in Don Summers' lab to do the necessary

polio experiments, and bring the RNA back to Glasgow for fingerprinting[20]. The poliovirus strain was an attenuated one and, as I recall, we did not need to handle it again in Glasgow. Nevertheless, JND and Martin Smellie rightly insisted that, for safety, all members of the department should receive polio vaccine; I had the congenial duty of administering sugar lumps treated with vaccine to the entire staff as they filed past!

Another problem concerned the absolute molar yields of the methylated oligos. Previously published data that related indirectly to this question were confusing. The solution came fortuitously when Salim and I visited Cambridge to learn some further separation methods from George Brownlee. George had been a student of Fred Sanger when I was with Robin Monro, and he was by this time a staff member. He mentioned a technique for characterizing oligo-A tracts in RNA by digesting the RNA using combined T_1 RNase (which cleaves after G residues) and pancreatic RNase (which cleaves after U and C residues). It was known that these enzymes would not cleave after 2′-*O*-methylated nucleotides. I realized that several rRNA oligos containing such 2′-*O*-methyls could be purified by electrophoresis from digests of ^{32}P-labelled rRNA and, therefore, quantified. Application of this method successively to ^{32}P-labelled and then [^{14}C]methyl-labelled rRNA gave us the required stoichiometries and, hence, for the first time, accurate values for the total numbers of methyl groups[21,22] – about 115 per human ribosome.

In 1972, Salim was awarded his PhD (Fig. 2); sadly, JND passed away shortly before this. In 1974, we published a comprehensive paper on this phase of the work[23]. Shah Khan had by that time joined the group, and we showed that rRNA-methylation patterns are highly conserved among vertebrates[24]. We were also directing our thoughts to the recognition processes that generate the many methyl groups present in different local sequence environments. One possibility was that some recurrent feature of secondary or tertiary structure was recognized, perhaps on the rRNA surface. To start to address this problem, Shah performed S_1-nuclease digests on methyl-labelled rRNA (Ref. 25). Contrary to our expectations, a wide range of sensitivites to S_1 nuclease was evident; this implied that some methylation sites were exposed but that others were buried in the three-dimensional structure.

While working on the problem of the absolute molar yields of the methylated oligos, we had established a friendly relationship with Rudi Planta's group in Amsterdam, who were doing very similar work on yeast rRNA. They showed that there were fewer methyl groups in yeast rRNA than in vertebrates[26], but that qualitative aspects (the many early ribose methylations and fewer late base methylations) were highly similar in yeast and vertebrates. We collaborated in the characterization of an oligonucleotide that contains a conserved, hypermodified nucleoside in 18S rRNA. The nucleoside, $m^1acp^3\psi$, had been identified earlier[27] and is remarkable because it incorporates label (in separate biosynthetic reactions) from the methyl group and then C1 of methionine[28].

All of this work was done before we had any means for determining the complete sequences of the rRNA molecules or the whereabouts of the methyl groups in the sequence. The methylated oligos were unplaced pieces in a linear puzzle. By this time, the *E. coli* 16S rRNA sequence was nearing completion in Strasbourg, but its assembly from the necessary partial digestions and overlaps had been a daunting task. We did not seriously contemplate applying such methods to human 18S or 28S rRNA. However, a revolution in methodology was about to occur.

Carnegie sabbatical

I had been following the Carnegie Embryology group's work on rRNA genes in *Xenopus laevis* with great interest. In 1969, I had paid them a brief visit. Later, in 1977–1978, I spent a sabbatical year at their lab. I was particularly fortunate for two reasons: (1) Carnegie was one of the leading centres at the beginning of the cloning revolution; and (2) there had been considerable competition for places there.

When I arrived, the large-scale structural characterization of ribosomal gene organization was essentially complete. Interest had moved to DNA sequencing (by the Maxam and Gilbert method) and, especially, to transcription. Don Brown's group were by that time concentrating on 5S RNA genes, while Ron Reeder's group were working on the genes for 18S and 28S rRNA (rDNA); this led to their ground-breaking work on transcription by RNA polymerases III and I, respectively.

The possibility of using rDNA to purify specific segments of rRNA for further analysis had been at the back of my mind. I was also considering the possibility that I could begin to localize the methyl groups by analyzing the RNA recovered after hybridization to specific segments of DNA. Don had electrophoresis tanks that I would be able to use for fingerprinting the recovered rRNA segments. I think both Don and Ron were surprised that I should wish to pursue this seemingly idiosyncratic (but intuitive) line of research rather than work on transcription. However, I was, and remain, very grateful that they agreed to my pursuing it. I made a start by determining which methylated oligos were recovered in RNA that hybridized to the left, and which were recovered in RNA that hybridized to the right, of the unique EcoRI sites in the 18S and 28S genes[29]. I also subcloned rDNA into smaller pieces, which would allow subsequent re-iteration of this general approach. Lastly, I learned the intricacies of the Maxam and Gilbert sequencing method from Ron Peterson, a postdoc who was working with Don Brown. These beginnings paved the way to the subsequent mapping of all but a few of the methyl groups.

Glasgow to Liverpool

When I returned to Glasgow in autumn 1978, I had a new student, Lucinda Hall. I was also able to arrange for Salim, who had been on the faculty at Islamabad for a few years, to spend a year on sabbatical with me. Lucinda set up the Maxam and Gilbert sequencing methodology and applied it to the difficult, GC rich, internal transcribed spacer region of *Xenopus* rDNA (Ref. 30). Salim acquired the new skills and sequenced the 18S gene. Meanwhile, I continued to map the 18S and 28S rRNA methyl groups to regions defined by smaller segments of rDNA. This work was technically demanding because the amounts of radioactivity recovered in [^{14}C]methyl experiments were quite low, and I often had to wait several weeks for good autoradiographs. I specialized in this technique but also familiarized myself with DNA sequencing.

By the time Salim had finished the 18S rDNA sequence, I had mapped the rRNA methyl groups to within tracts of a few hundred nucleotides. In most instances, we had sufficient sequence data from the earlier work on the methylated oligos[23] to identify a unique sequence in the relevant tract of rDNA defined by the hybridization experiments. We could therefore pinpoint the RNA methyl groups in the complete sequence derived from rDNA. This work was published in

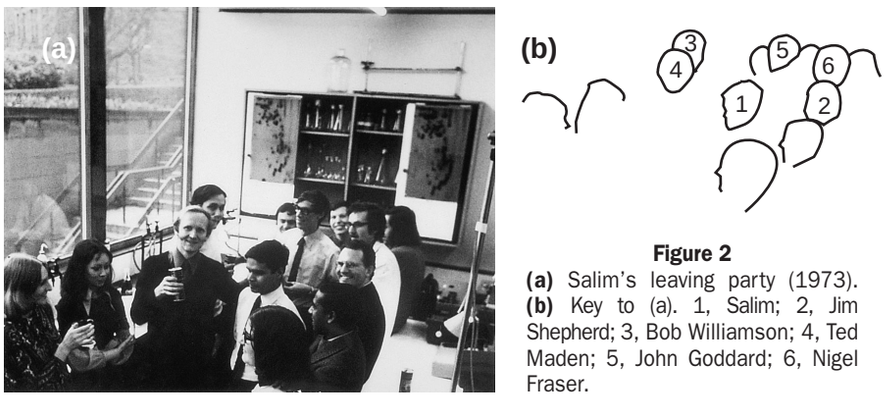

Figure 2
(a) Salim's leaving party (1973). **(b)** Key to (a). 1, Salim; 2, Jim Shepherd; 3, Bob Williamson; 4, Ted Maden; 5, John Goddard; 6, Nigel Fraser.

1981[31]. (A single correction to the DNA sequence was made later[32].)

In 1983, I became a professor at the University of Liverpool. At this time, rRNA sequences, usually derived from the genes, were accumulating rapidly in the literature, and consensus models for rRNA secondary structures were emerging. Knowledge of the methyl-group locations was becoming increasingly important in this structural context.

I worried that locating the 18S methyl groups had required a large amount of evidence, which had taken many years of research to accumulate, and that it had not been possible or appropriate to give that evidence in detail in our *Nature* paper[31]. I rectified this in 1986, by writing a paper that incorporated all the data from our own lab, as well as all relevant published data from other labs[32]. Then followed the even-more daunting task of assembling the 28S data (we still had some unassigned methyl locations), and this was published in 1988[33]. A review of our work and that of others, including the problem of locating pseudouridines, on which only limited progress had been made at that time, was published in 1990[34]. Later, both we and Jim Ofengand's group developed the reverse-transcriptase methodology, which facilitated location of pseudouridines[35] and 2′-*O*-methyl groups[36].

A puzzling aspect of these modifications was that only one research group, Douane Eichler and colleagues, reported any success in obtaining site-specific ribose methylation *in vitro* – and this was confined to a triplet of methyl groups in 28S rRNA (Ref. 37). Denis Lafontaine and colleagues[38] isolated the gene that encodes the enzyme that modifies the two base-methylated dimethyladenosines near the 3′ end of the 18S rRNA.

The finding that snoRNAs direct modification (ribose methylation and pseudouridylation) of the target nucleosides[1–4] has clarified immensely the recognition processes that underlie what had seemed to be a bewildering diversity of modification sites. Moreover, the discovery of the involvement of snoRNAs, particularly the finding that many snoRNAs in higher organisms are derived from introns[2], has linked rRNA modification to a much wider field of molecular cell biology.

The rRNA methylation work described here paralleled general advances in our understanding of ribosome structure and biosynthesis, as well as of modification of other RNA molecules – tRNA, snRNA and eukaryotic mRNA. In 1994, an EMBO workshop on RNA modification and editing, planned by Henri Grosjean with help from Glenn Björk, James McCloskey and myself, was held in Aussois and was deemed highly successful in bringing together many different workers and points of view. The proceedings were published in *Biochimie* (Vols 76 and 77), and a comprehensive book, *Modification and Editing of RNA*, which was conceived at the workshop, has recently been published[39].

Note

The early conclusion that pre-rRNA methylation is essential for ribosome maturation[13] requires, and is receiving, further work. In general, ribosome maturation is not abolished in the single-methyl-group knockouts that are now available. The current, rapidly accumulating evidence is beyond the scope of this article, but is reviewed elsewhere[1,2,5].

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