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SESSION 1: mRNA PROCESSING

Biochemical Identification of Nuclear Proteins p30 Specifically Interacting with Upstream Repressive Element of Adenovirus L1 Poly(A) Site

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Alternative polyadenylation in adenovirus is a temporal selection process involving five poly(A) sites usage along viral infection course. During early viral infection, viral transcripts encoding structural proteins are low and are only processed at the first three poly(A) sites (L1 to L3) with the predominant cleavage at the promoter proximal L1 site. However, after viral DNA replication, all five poly(A) sites are highly utilized and equally represented in the late stage of infection. Using minigene system, an upstream repressive element (URE) locating at upstream of L1 poly(A) site has been shown to be a novel modulator to control both gene expression and poly(A) switch. In this study, biochemical analysis was performed to identify if specific nuclear factor(s) can interact with URE. Unexpectedly, in the absence of UV irradiation, a set of RNase A resistant complexes with molecular weights around 30 kD were covalently linked to L1 poly(A) site. The formation of the RNase A resistant complex is dependent on two defined sub-effectors within URE, abolishing any of the effectors could prevent the complex formation. In addition, the complex formation is independent on early or late viral infection and was sensitive to SDS treatment. Using *in vitro* synthesized URE as a probe, two proteins with MW>30 kD could be purified from cell nuclear using affinity column. In summary, this study clearly demonstrated that specific nuclear factors can interact with URE *in vitro*, and this interaction may play an important role in controlling Ad gene expression and poly(A) switch.

C-Terminal Deletion Analysis of Mouse Capping Enzyme

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Mouse capping enzyme (MCE) is consisted of two functional domains: the amino-terminal triphosphatase domain and the carboxyl-terminal guanylyltransferase domain. The bifunctional MCE1 gene encodes a 597 a.a. protein with a molecular weight>68 kD. Studies indicated that both the wild type of MCE as well as its truncated form without first 211 a.a. can complement the null mutation of yeast capping enzyme (Ceg1). In order to define the C-terminal border of MCE and to isolate the minimal and functional domain of MCE, in this study, four truncations were produced by deleting 2, 30, 37, and 60 a.a. from the C-terminus of either the wild type (MCE-WT) or the shorter 211 truncation (211-597R). Total eight deletion constructs were generated and subcloned into the yeast pYX plasmid. Using counterselection approach, it has been demonstrated that the minimal and functional domain of MCE is between the amino acid 211 and 567. In order to detect the function of those deletion mutants, the truncations were also expressed in BL21-pET system. Purified proteins were assayed for their guanylytransferase activities. In consistent with yeast genetic study, both 211-567R and 211-585R had significant guanylytransferase activity, while 211-537R and 211-560R were inactive. Overall, both *in vivo* and *in vitro* studies indicate that lacking 30 a.a. from C-terminus does not prevent MCE enzymatic activity.

SESSION 2: RIBOZYME & RNA FOLDING

GAPDH Induces Group II Intron Splicing *in Vitro*

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Group II introns are autocatalytic RNAs, which self-splice *in vitro* under high, non-physiological salt conditions. However, under *in vivo* low salt conditions additional protein factors have to be involved in the splicing process. We used a *vitro* affinity chromatography method called "StreptoTag" [Bachler *et al*., RNA, 1999, 5: 1509] in order to find group II intron binding proteins from *Saccharomyces cerevisiae*. This simple method uses a hybrid RNA consisting of a streptomycin-binding aptamer and the RNA of interest. This RNA is bound to a streptomycin column and incubated with yeast mitochondria protein extracts. After several wash-steps the specifically bound RNP complexes are eluted by addition of streptomycin. The eluted RNPs are separated and identified with SDS-PAGE and subsequent mass-spec

analysis. Using crude mitochondria extract from yeast in combination with a substructure of the bI1 group II intron (domains 4–6) we were able to identify 4 glycolytic enzymes as putative group II intron binding proteins. These are glucose-6-phosphate isomerase (GPI), 3-phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI). From these proteins, GAPDH can increase *in vitro* splicing of the yeast mitochondrial group II intron bI1 up to 15%, whereas the other three identified proteins failed to do so. This is another example of an RNA-GAPDH interaction besides its important role in glycolysis. Further studies will enlighten a possible role of GAPDH in group II intron splicing *in vivo*.

Hammerhead Ribozyme Mediated Suppression of the Survivin Expression for Cancer Gene Therapy

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Aims Both tumor specific over-expression and the antagonizing role against the mitotic disorders as well as apoptosis have made the surviving [a newly identified member of the IAP (inhibitor of apoptosis) family] an attracted target for anti-cancer efforts via genetic manipulation. Methods: In this report, we have used a pol III promoter based new-generation vector pGVaL to construct and test five hammerhead ribozymes both in test tube and in cell cultures where both retroviral and adenoviral vectors were employed as the delivery vectors, respectively. Results: We found two [R1 (+61) and R3 (+232)] of five (at the following sites of +61, +83, +232, +294 and +358 in the survivin mRNA) capable of cleaving the survivin RNA substrates in test tubes as well as repressing expression of the endogenous survivin at both mRNA (by a semi-quantitative PCR) and protein (by Western analysis) levels in a hepatocellular carcinoma cell line, BEL-7402. Such effects correlate well with the reduced cell growth (via the MTT based cell proliferation assay) and increased apoptotic population of cells (with the increased level of apoptotic marker, PARP p85 protein). **Conclusions** Our efforts would provide the ribozyme based tools for unveiling the underlying mechanism whereby the survivin maintains the mitotic integrity and antagonizes the apoptosis of the tumor cells as well as the potential genetic remedies for the malignant lesions.

Rapid Folding of the Native P3-P7 Core Determines the Entry to the Trap-Free Pathway

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The *Candida* ribozyme tends to fold to the catalytically active structure without being trapped in energetically frustrated intermediates, providing an idea model to study the folding events in the trap-free pathway. The folding of the native P3-P7 core was explored in this study. Catalysis-measured folding experiments demonstrated that the native P3-P7 core folded rapidly and required $Mg^{2+}\geqslant$1.5–2.0 mM, which is coincident with the rapid folding of the compact overall structure of the ribozyme. As revealed by the gel-shift analysis, magnesium- mediated rapid folding of the native core quantitatively agreed with the catalytically active structure folded in a later time. The rapidly populated misfolded intermediates containing the non-native P3-P7 structure were hardly converted to the active ribozyme. The lack of a stable Alt P3 and the minimal mispairing in the most stable structure predicted by energy minimization explains the trap-free folding of the major population of the ribozyme. These findings shed new light on the role that the self-consistency may play in the cooperative folding of the large, multidomain RNA.

Key words RNA folding; RNA structure prediction; folding pathways; metal ion

Sequential Folding Slows the Formation of the P3-P7 Core of the *Candida* Ribozyme

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Our previous study has shown that $Mg^{2+} \geqslant$ 1.5–2.0 mM is required for rapid folding of the native P3-P7 core of the purified *Candida* ribozyme, eliminating the binding of the antisense oligos pairing the continuous strand of the core and any inhibition of the ribozyme activity. The *in vitro* transcription-initiated folding of the P3-P7 core was studied in this work. When the oligo was added to the reactions transcribing the trans-acting ribozyme and precursor in the presence of 6 mM Mg^{2+}, we surprisingly found that potent inhibition of the ribozyme catalysis was detected. RNase H assay in those reactions demonstrated that the antisense oligo readily bound to the unformed P3-P7 region, explaining the catalytic inhibition. Therefore, folding of the P3-P7 core of the *Candida* ribozyme during transcription is slower than that during refolding the purified ribozyme, in consistent with the lower splicing activity observed during transcription. Therefore, consistent with the idea that folding of the P3 and P7 helices involving long-range interaction is limited by the availability of the later transcribed strand, sequential folding of the group I ribozyme is not necessary to favor the native structure.

Key words sequential folding; refolding; group I ribozyme

Non-conserved P2.1 Stabilizes the Tertiary Structure and Slows the Native Folding of the *Candida* Ribozyme

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Non-conserved structural elements of group I ribozyme may contribute to the folding of these RNA molecules. The role of a non-conserved peripheral element P2.1 of the *Candida* ribozyme in the ribozyme folding was studied. The mutant ribozyme $E\Delta^{P2.1}$ with P2.1 deleted required

over 15-fold more magnesium than the wild type ribozyme to reach the half maximal cleavage of a small substrate RNA measured by k_{obs}. The increased Mg^{2+} concentration fully restored the catalytic activity of $E\Delta^{P2.1}$ compared to the ribozyme, suggesting that P2.1 plays an essential role in stabilizing the catalytic structure of the ribozyme. Consistent with this conclusion, T1 footprinting analysis revealed that about 9-fold magnesium was required to fold the overall all structure of the mutant ribozyme to the half protected state as compared to the wild type ribozyme; the tertiary structure of the core region of the mutant ribozyme requires much more magnesium. Surprisingly, although the maximal cleavage of the ribozyme was lower, $E\Delta^{P2.1}$ folded to the catalytically active structure 5-fold faster than that of the wild type ribozyme in the presence of 100 mM magnesium, suggesting that the stabilization of the ribozyme by P2.1 at high magnesium concentrations opposes the native folding of the ribozyme.

Secondary Structure Is Essential in Native Folding of a Large RNA

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Tertiary folding of a large RNA is believed to follow the formation of the secondary structure. We used the trans-cleavage activity of the *Candida* ribozyme as a readout for native tertiary structure to investigate the effect of the secondary structure on RNA folding. Na^+ and $Tris^+$ are known to support the formation of the RNA secondary structure, while Mg^{2+} and Ca^{2+} support the tertiary folding. Using the refolding in H_2O as a control, refolding of the ribozyme RNA in the presence of Na^+ or $Tris^+$ prior to the cleavage reaction containing 10 mM Mg^{2+} significantly increased the cleavage rate of the ribozyme while slightly increased the maximal cleavage. In contrast, the presence of low concentrations of Mg^{2+} increased the cleavage rate at a cost of decreasing the maximal cleavage; refolding at high concentrations of magnesium eliminated the cleavage activity, consistent with the observed aggregation of the ribozyme RNA under this condition. Compared with the samples refolded in the presence of these cations, UV absorption of the ribozyme RNA in H_2O was significantly higher, indicating that the ribozyme is largely in the unfolded states in H_2O. These results are consistent with the explanation that Na^+, $Tris^+$, and low concentrations of Mg^{2+} support the formation of the secondary structures of the ribozyme, facilitating the followed tertiary folding of the ribozyme RNA. In contrast, initiating RNA tertiary folding without native secondary structures tends to result in the non-native structures, providing evidence that the native secondary structure is necessary for the formation of the native tertiary structure of a large RNA. Furthermore, the presence of Na^+ and $Tris^+$ in the direct cleavage reaction significantly increased the ribozyme activity (k_{obs}), and the concentrations required to reach the half maximal activity increased up to 3-fold when the concentration of Mg^{2+} increased from 10 mM to 15 mM, further proving the importance of the secondary structure in the RNA tertiary folding.

Phylogenetic Distribution of Group I Introns in rRNA Genes

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Group I introns are found in many lower eukaryotes and a few bacteria. Structural analysis reveals 13 subgroups of these introns classified based on the structural and sequence conservations. In this work, 1674 group I introns identified in the rRNA genes were collected from GenBank, and the hosts' species, intron subgroup and insertion position (IP) of all these introns were analyzed. Except for one intron in the rRNA gene of a bacterium, all others were found to cluster in lower eukaryotes, with 66.4% in fungi, 19% in viridiplantae and 9.6% in rhodophyta. Among the 1168 introns with defined subgroups, 61.1% belong to subgroup IC1 and 18.7% to IE. 1.9%–5.1% of introns fall into IC2, IB, IA3, IB4 and IA1 subgroups, and none or a few introns belong to each of the other 6 subgroups. Hence, most introns from the rRNA genes of the lower eukaryotes adopt a limited structural entity (IC1 and IE class). Over 83% of the defined group IE introns were from fungi; 94.1% of the group IA3 introns and 94.4% of the group IB4 introns were from viridiplantae. Rhodophyta contains only group IC1 introns. These findings suggest a strong correlation between the intron structure and host species, which may reflect the sequence characteristics of the adjacent exons of the rRNA precursor and/or variation in the protein factors required to assist the intron folding and splicing in these organisms.

Key words group I introns; phylogenetic distribution; rRNA gene

Native Secondary Structures of All Group I Introns in the Subgroup E

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Group I introns exist exclusively in lower eukaryotic organisms. They can form complex catalytic structures, and catalyze their own excisions from the precursor RNAs. Sequence analysis in the 1980s revealed the common secondary structure shared by all Group I introns. This secondary structure model includes 9 conserved paired regions. 2 of the 9 paired regions form a pseudoknot, which is essential for the catalytic activity of Group I introns. The introns are further classified into 12 subgroups based on their sequence and secondary structure features (Michael & Westhof, 1990), designated A1 to E. Currently, the sequences of over 1700 Group I introns are available in databases. Hampered by the existence of the pseudoknot, the drastic variation of sequence and the length of the intron, no satisfactory computational method for predicting the native secondary structure of a group I intron has been developed. Compared to the rate that sequences are depositing into the databases, the process in secondary structure analysis significantly lags behind

In this work, we have analyzed the sequences of 212 group I introns belonging to the subgroup E (group IE) using a manual comparative sequence analysis method. The P7' regions were first located by searching with their highly conserved sequences. Then the adjacent J8/7, P8,

P3', P7, P9 regions were recognized. The more distant regions (P3, P4, P5, P6) were identified by their relative distance to P7 and characteristic sequences. P1 were recognized by the conserved sequence. The recognized regions hence divided the sequence into small segments, which were usually shorter than 80 nt and included simple secondary structures that were readily predicted by the software *RNAstructure*. As a result, the secondary structure of each group IE intron was generated, which allowed the production of the structural alignment for the whole subgroup E. The secondary structures and alignment were further refined iteratively. The uniformity of the sequences in the core regions of the group I introns showed that the final result of the structure prediction and aligning was quite reliable. This secondary structure-based alignment gives clues to the structural and catalytic importance of each component of the introns. The details of the structural information and phylogenetic relation of the group IE introns will be discussed.

Comparative Sequence Analysis Methods Developed for RNA Structure Prediction

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Knowledge of the secondary structure of biological RNAs is essential in understanding their functions. Many computational methods for prediction of the RNA secondary structure have been developed. Comparative sequence analysis has been proven to be a very efficient tool in determining the RNA secondary structure and many tertiary interactions. In this work, two main programs, based on the algorithms of the comparative analysis methods, were developed and a systemic route for RNA secondary structure and tertiary structure prediction were developed. One program was developed to compute the covariation information and compensation value that defines RNA base pairing of a given alignment. The other was to compute the information of base-triples representing RNA tertiary structure of an alignment. The scores for each potential base pair were then sorted and integrated, and the most reliable base pairs and base triples as a part of the final structure were then choose to deduce the secondary and tertiary structure. The programs were successfully used to deduce the secondary structure and tertiary structure of a given tRNA based on an alignment of 50 tRNA sequences. Comparison of the resulted structures with those from the previous work validated the accuracy and efficiency of the method. The structure of the group I intron *Pc. SSU* containing pseudoknot was successfully deduced using the alignment of 87 sequences of group I introns. Hence, the method proposed in this work is reliable for RNA structure prediction and requires less manual efforts.

Key words RNA secondary structure; tertiary structure; comparative sequence analysis; mutual information; covariation

Ssh10b, a Conserved Thermophilic Archaeal Protein, Binds RNA *in Vivo*

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Proteins of the Sac10b family, which is highly conserved among hyperthermophilic archaea, have been regarded as DNA binding proteins. Based on their *in vitro* DNA-binding properties, these proteins are thought to be involved in chromosomal organization or DNA repair/recombination. Ssh10b, a member of the Sac10b family from *Sulfolobus shibatae*, bound with similar affinities to double-stranded DNA, single-stranded DNA and RNA *in vitro*. However, the protein was exclusively bound to RNA in *S. shibatae* cells, as revealed by *in vivo* UV-crosslinking and coimmunoprecipitation. Ribosomal RNAs were among the RNA species coimmunoprecipitated with Ssh10b. Consistent with this observation, Ssh10b was co-purified with ribosomes under low salt conditions. Furthermore, we demonstrate by UV-crosslinking hybridization that, when the cells were irradiated with UV, Ssh10b became crosslinked to 16S, 23S rRNAs and mRNAs. Because of its high abundance in the cell it is speculated to be an important protein binding with rRNAs and mRNAs widely. No specific mRNA signal was detected in the total RNA from *Methanococcus maripaludis*, a moderate archaea whose genome encodes the homologue of Ssh10b. Thus the Sac10b family may belong to hyperthermophilic archaea. The knowledge of the binding targets of Ssh10b is the important basis of further studying the physical function of the Sac10b family.

Key words Ssh10b; Sac10b family; RNA-binding protein; archaea

SESSION 3: RNOMICS & SMALL RNAS

Rnomics In Yeast: A Large Scale Screen for SnmRNAs in *Schizosaccharomyces pombe*

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An experimental RNomics approach has been applied successfully to different model organisms such as the mouse *Mus musculus*, the plant *Arabidopsis thaliana*, the archaeon *Archaeoglobus flugidus* and the fruitfly *Drosophila melanogaster*. Yet study of RNomcis in yeast has not been reported. Therefore we took initiative to construct a specialized cDNA library and screen for snmRNAs on a large scale in *S. pombe*.

The cDNA library was generated with small RNAs sized from about 50 to 500 nt in order to investigate the population of snmRNAs in *S. pombe*. 100 clones from this library were randomly sequenced and identified by a BLASTN database search. Most of the clones could be assigned into genes encoding known snmRNAs, of which 5.8 S, 18S and 25S rRNA gene fragments accounted for 23%, 29% and 12%, respectively, and the U snmRNAs for 5%. In addition, 10% of the sequences were derived from known or hypothetical mRNA and pre-mRNA fragments. However, only 6% of cDNA sequences were not announced previously in GenBank and hence represented potential novel snmRNAs. From 3000 clones screened by hybridization procedure, 400 clones exhibiting low hybridization signals were sequenced. By this

approach, the amount of novel RNA species could significantly increase from 6% to 28%. However, there were still about 35% clones carrying degradation fragments from rRNA genes in our analysis, probably due to the fact that many of these fragments were too short to be overlapped efficiently by probes. A total of 110 sequences were not mapped to the announced genes and therefore considered as potential candidates for novel snmRNAs. More than 60 of those snmRNA candidates exhibited hybridization bands on northern blot analysis, suggesting they are stable novel snmRNAs. Among the novel snmRNAs confirmed by Northern blotting, 28 species belong to family of C/D box snoRNA and 20 species to that of H/ACA snoRNA. The remaining 12 species belong to the class of snmRNAs lacking known sequence and structure motifs. This study sets the stage for the functional analysis of all snmRNAs identified in our library.

Key words RNomics; snmRNA; snoRNA; cDNA library

SP20, a Novel SnoRNA with an Unexpected Gene Organization in *Schizosaccharomyces pombe*

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Through screening cDNA library, a novel snoRNA, SP20, was identified from *Schizosaccharomyces pombe*. Its secondary structure resembles the canonical H/ACA box snoRNA, which consists of two large hairpin-like domains linked by a single-strand hinge and followed by an ACA-like tail. However, instead of ANANNA motif, a noncannonical H box(AGAGCC) was found in the hinge region. Based on the relationship between structure and function of the box H/ACA snoRNAs, SP20 is predicted to guide the pseudouridylation of U1208 and U1053 on *S. pombe* 18S rRNA respectively. More interestingly, SP20 gene is nested inside a very hypothetical ORF(SPBC1921.04c), but transcribed in a reverse sense. Northern blot and RT-PCR analyses could not detect the transcript from the hypothetical ORF, and therefore excluded the expression of the spurious gene. This is the first example that a genetic locus predicted for a protein-coding gene actually encodes a small non-messenger RNA (snmRNA) in *S. pombe*. Our findings suggested that some snoRNA genes might be located in annotated protein-coding regions of genome rather than the introns only. As a result, snoRNA searches limited to annotated non-coding regions are unlikely to yield per se the complete set of snoRNAs within a given organism.

Key words box H/ACA snoRNA; pseudouridylation; gene organization; *Schizosaccharomyces pombe*

Identification and Characterization of MicroRNAs in *Oryza sativa*

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MicroRNAs (miRNAs) are a large family of noncoding RNA of 20–25 nt whose function is to regulate gene expression in post-transcription. These RNA molecules of 20–25 nt are complementary to the 3'-untranslated regions (UTRs) of target mRNA and direct negative post-transcriptional regulation through RNA duplex formation. The first endogenous about 22 nt RNA was lin-4 and let-7, both of which were key regulatory molecules in the pathway controlling the timing of larval development in the nematode *Caenorhabditis elegans.* Recent studies revealed that more than 200 genes of microRNAs have been identified in worms, flies, animals and plants. They were processed from longer precursor RNA that formed stem-loop hairpin structures by the ribonuclease RNase III, Dicer and Argonaute, which were thought to regulate gene expression by base pairing with RNAs of protein-coding genes. In animals, miRNAs are typically excised from 60–70 nucleotide fold-back RNA precursor structures, whereas in plants they are more variable, and may include up to a few hundred nucleotides, which are sometimes detected at the onset of miRNA precursor expression or during the expression of very abundant miRNA. Generally, only one of the strands of the hairpin precursor molecule is excised and accumulates, presumably because it is protected by associated proteins from RNA degradation.

Here we described the identification of novel miRNAs by size-fractional RNA from *Oryza sativa*. 229 tiny RNAs were cloned from the seedling, embryo, leaf, stem and root of *Oryza sativa*. Of these, 22 sequences were represented by more than one clone. 195 unique, putative small RNA sequences were subjected to BLAST analyses against *Oryza sativa* genome. Among the 195 sequences identified, most arose from intergenic regions, as well as from intron, 3' UTR and coding region of mRNA. Interestingly, most of the rice miRNAs begin with a U, a trend previously observed in animal miRNAs and in *Arabidopsis* miRNAs. A total of 126 sequences from 19–27 nt in length were further analyzed to predict secondary structures by the m-fold program. One of the sequences had complete similarities to an miRNA of *Arabidopsis*, which had been named miR39, with gene designated MIR39. Each of these 51 miRNA loci places the cloned RNA sequence in a context where it can pair with a nearby genomic segment to form a potential fold back precursor structure that contains the 20–25 nt sequence within one arm of the hairpin. 21 putative miRNAs have been confirmed by Northern hybridization, which occur in *Oryza sativa* embryos, leaves, roots and stems, respectively, suggesting that the miRNAs could play a regulatory role in the development of plants as well as animals.

Plant snoRNA Project: Systematic Analysis of BoxC/D snoRNA Genes from *Oryza sativa*

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SnoRNA (small nucleolar RNA) plays an important role in ribosome biogenesis. Since the 1990s, a myriad of novel snoRNAs have been identified and characterized in the nucleolus of eukaryotic cells, most of which came from vertebrates and yeast. In plants, although early biochemical data had revealed their highly methylated rRNAs, only very recently was the first comprehensive study of box C/D snoRNA genes carried out with the *Arabidopsis* genome.

Knowledge of plant snoRNA genes continues to expand, particularly with the progress of the Rice Genome Project. The genome of rice is

four times larger and more complex than that of *Arabidopsis*. In our initial study of the rice genomic DNA sequences in the database, we discovered a new mode of organization, i.e. intronic snoRNA gene clusters in plant. A more comprehensive study of snoRNA gene content and organization from rice as well as other plants will be important to clarify the plant rRNA methylation profile and to understand the strategy of adopting novel modification sites in plant rRNAs and its biological meaning. In this regard, we took advantage of the recently published draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*) to perform a large-scale search for snoRNAs with the powerful computer-assisted analysis strategy. A large number of rice snoRNA genes have been identified and compared with those of other organisms, especially *Arabidopsis*. Detailed analysis has also been performed on gene organization and sequence variation of rice snoRNAs to find clues about the mechanisms of the duplication and functional evolution of snoRNA genes in plants.

This analysis identified 120 different box C/D snoRNA genes with a total of 346 gene variants, which were predicted to guide 135 2'-O-ribose methylation sites in rice rRNAs. Though not exhaustive, this analysis has revealed that rice has the highest number of known box C/D snoRNAs among eukaryotes. Interestingly, although many snoRNA genes are conserved between rice and *Arabidopsis*, almost half of the identified snoRNA genes are rice specific, which may highlight further the differences in rRNA methylation patterns between monocotyledons and dicotyledons. In addition to 76 singletons, 70 clusters involving 270 snoRNA genes were also found in rice. The large number of the novel snoRNA polycistrons found in the introns of rice protein-coding genes is in contrast to the one-snoRNA-per-intron organization of vertebrates and yeast, and of *Arabidopsis* in which only a few intronic snoRNA gene clusters were identified. Furthermore, due to a high degree of gene duplication, rice snoRNA genes are clearly redundant and exhibit great sequence variation among isoforms, allowing generation of new snoRNAs for selection. Thus, the large snoRNA gene family in plants can serve as an excellent model for a rapid and functional evolution.

Key words *Oryza sativa*; genome; snoRNA; gene cluster

snoRNA Isoforms from *Oryza sativa* Show Clues of Gene Evolution

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Rice has the highest content of box C/D snoRNAs among eukaryotes and two thirds of them have two or more isoforms through duplication of gene clusters on the same or different chromosomes or tandem gene duplication within a cluster. Thus mutations can be greatly tolerated due to this gene redundancy of rice snoRNAs. Sequence comparisons of different isoforms virtually reveal numerous sequence changes and small insertions and deletions. Although most changes are insignificant, accumulation of certain mutation alters the function of some snoRNA genes and produces novel rRNA complementary sequences that could lead to novel methylation sites under selection. Isoforms of snR39BY, U33 *et al*. have gained or lost one functional region due to nucleotide mutations. The upstream antisense element of different isoforms of snoR137 probably shows a step-by-step course of how mutations gradually affect a functional region including some intermediates. Moreover, mutations can cause one of the isoforms to target the same site as the other snoRNA gene although other mechanisms might be also in action. Finally, that with one functional region fixed the other can evolve freely for new RNA targets without risking invalidation of the whole snoRNA molecule might partially explain the advantages of possessing two antisense elements.

Identification and Characterization of Two Novel U24-Like snoRNA Genes from Fission Yeast

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Two novel box C/D snoRNAs were identified from *Schizosaccharomyces pombe* by screening a specialized cDNA library generated from nuclear small RNA. The two snoRNAs each possess one of the two antisense elements of budding yeast U24 snoRNA and therefore were designated as U24 and U24b, respectively. Northern blot and reverse transcription analyses of total cellular RNAs showed that U24 and U24b, being 76 and 83 nt respectively, were expressed stably in *S. pombe*.

In fact, *S. pombe* U24 snoRNA possesses two antisense elements that are complementary to two closely spaced regions of 25S rRNA. The downstream antisense element (DAE), which is functionally equivalent to the DAE of budding yeast U24 snoRNA, exhibits perfect complementarity to a conserved region of 25S rRNA at position 1468–1477. The upstream antisense element (UAE) pairs perfectly to the region of 25S rRNA at position 1483–1492. *S. pombe* U24b snoRNA possesses only a fourteen nucleotides long UAE to 25S rRNA at position 1479–1492, which is functionally equivalent to UAE of budding yeast U24 snoRNA. Using the dNTP concentration-dependant primer extension assay, we identified only two methylation sites, Cm1471 and Am1483, in *S. pombe* 25S rRNA. The site A1486 guided by upstream antisense element of U24 was found to be unmethylated in our experiment, suggesting that this guide region is probably involved in cleavage of pre-RNA or RNA fold by mechanisms that have not yet been detailed.

Both *S. pombe* U24 and U24b are intronic snoRNAs, encoded in 3' UTR of a hypothetical protein gene and a guanine nucleotide-binding protein β subunit-like protein gene, respectively, while in *S. cerevisiae* U24 is intron-encoded in a guanine nucleotide-binding protein gene. This result provided an important evidence for diversification of structure and genomic organization of snoRNA in two yeasts.

Key words snoRNA; U24; 2'-O-methylation; fission yeast

Plant snoRNA Genes: Polycistrons are Better than Singlets

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Small nucleolar RNAs (snoRNAs) are a well characterized family of non-coding RNAs (ncRNAs) and widespread in eukaryotes even in

archaea. The two major classes of eukaryotic snoRNAs are the box C/D and box H/ACA snoRNAs. The former acts as guide RNAs determining the sites of 2'-O-ribose methylation of rRNAs, while the box H/ACA snoRNAs are involved in rRNA pseudouridylation. The diversity and complexity of this gene family continue to expand with the discovery of scaRNA and orphan snoRNAs that target snRNA or other unknown RNAs.

It has been shown that plant rRNAs are highly modified. Recently, genomic analyses of two plants, *Arabidopsis* and rice, revealed that plants have the highest number of known snoRNAs among eukaryotes. Remarkably, plant snoRNA genes are distinct from those of vertebrates and yeast in their organization and mode of expression. All of vertebrate and some of yeast methylation guide snoRNAs are intron-encoded by protein-coding or ncRNA genes. The mode of one snoRNA-per-intron is strictly maintained if a host gene encodes multiple snoRNAs. However, most plant snoRNAs are arranged in two types of polycistronic organizations, that is, gene cluster and intronic gene cluster. Both gene clusters are transcribed from an upstream promoter into a polycistronic snoRNA precursor, which is processed by endonucleolytic and exonucleolytic activities to release each mature snoRNA. Plant snoRNA polycistrons are usually composed of one type of snoRNAs such as box C/D snoRNAs or box H/ACA snoRNAs. In few cases, heterogeneous snoRNA clusters were observed. Interestingly, recent analysis demonstrated that tsnoRNA clusters comprise a tRNA and a snoRNA as a transcriptional unit and from which the dicistronic transcript is processed by RNase Z. On the other hand, yeast and vertebrate usually contain few snoRNA gene variants, whereas the majority of plant snoRNA genes is present in a number of copies and exhibits great sequence variation among isoforms that may reflects the events of duplication and chromosomal rearrangement in plants.

Now, large numbers of plant snoRNA genes are available in the plant snoRNA database. The snoRNA gene family in plants can serve as an excellent model for rapid and function evolution.

Endogenous Small RNA in Rice

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Eukaryotes have several types of tiny regulatory RNAs that play crucial roles as regulators of gene expression. MicroRNAs (miRNAs) are small noncoding RNA gene products about 22 nt long with a 5'-terminal monophosphate and 3'-terminal hydroxyl group, which are found in diverse organisms, plants, invertebrate and vertebrate. They arise from the precursor RNA which has stem-loop structure. MiRNAs can repress translation of mRNA by near complement to the RNA sequence. miRNA is remarkably similar to small interfering RNA (siRNAs) which are the 21–25 nucleotide double strands that mediate RNA interference.

Small RNAs in 15–25 bp in seedling and young spikelet of 93-11 (*Oryza sativa.* L) were cloned by cDNA clone of sequence from size-fractionated RNA samples. 27 sequences of small RNA were got by sequence of 80 samples, 12 of which are rRNA and 1 is tRNA, the other 14 sequences were analyzed with m-fold on line. 6 sequences with the stem-loop structure were proven further by Northern blot hybridization, finally two miRNAs were found. One lies in two introns of putative serine/threonine-specific protein on chromosome 1. The other locates in the intergene region, and is expressed only in seedling.

Key words miRNA; *Oryza sativa*; small RNA

Isolation of Specific sncRNA Binding Proteins Using the Streptomycin Binding RNA Aptamer as an Affinity Tag

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Over the past few years a large number of small non-coding RNAs (sncRNAs) have been discovered that were not detected by traditional gene finding approaches. Bacterial sncRNAs play important roles in processes like the cellular response to environmental stress by regulating translation of their target mRNAs. Some sncRNAs affect bacterial virulence and others encoded by plasmids and phages have been shown to regulate replication and phage development. Eukaryotes small RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs), are key components of an evolutionarily conserved system of RNA-based gene regulation. The action of these sncRNAs is modulated by cellular protein factors like the bacterial protein Hfq which is required for the function of many of the around 50 sncRNAs that have been identified in *E. coli* so far. We use *E. coli* as a model system to find other protein factors that might be involved in the activity of sncRNAs. RNA hybrid constructs containing the Streptomycin binding aptamer and several sRNAs from *E. coli* were designed. We used a variety of RNAs including MicF, DsrA, DicF, OxyS, CsrB, RprA, Q1a, RhyB, 6S and Sp42. These RNA hybrid constructs were bound to a Streptomycin column and tested against *E. coli* total protein extract prepared under different conditions and growth phases. Proteins specifically eluted with streptomycin were identified by mass spectrometry using peptide mass fingerprinting and peptide sequencing. In addition to Hfq a series of other RNA binding proteins were found to associate with these RNAs and analysis of the specificity of these interactions is currently under way.

A Novel snoRNA Can Direct Site-specific 2'-O-Ribose Methylation of snRNAs in *Oryza sativa*

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Small nucleolar RNAs (snoRNA) are a kind of noncoding RNA, and the majority of snoRNAs is involved in site-specific modifications of

rRNAs. A novel box C/D snoRNA called snoR124 was found in *Oryza sativa*, and it can direct 2'-O-ribose methylation of spliceosomal small nuclear RNAs (snRNAs). The snoRNA has two antisense elements, and the results of primer extensions at different dNTP concentrations provide evidence that snoR124 guide 2'-O-methylations of C93 in the U4 snRNA and the T87 residue in the U5 snRNA. In addition, this snoRNA is located in a snoRNA gene cluster with another 7 snoRNAs that are identified to direct ribose methylation in rRNAs. This is consistent with the opinion that the snoRNA gene organization in plant is mainly gene cluster. The snoR124 is the first example of a snoRNA that directs modifications of RNAs other than rRNAs in plant; it will avail to get more insights into the function of snoRNAs in plant.

Key words 2'-O-ribose methylation; box C/D snoRNA; snRNA; *Oryza sativa*; plant

SESSION 4a: siRNA & APPLICATION

Activation of the Protein Kinase PKR by Small Double-stranded RNAs Flanked by Single-stranded Regions

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The human RNA-activated kinase PKR is an interferon-induced protein that protects the host against viral infection. PKR activity involves RNA-dependent autophosphorylation and RNA-independent inhibition of translation. PKR has an N-terminal dsRNA-binding domain (dsRBD) that can interact with long (>33 bp) stretches of dsRNA, leading to activation. In addition, certain viral and cellular RNAs containing non-Watson-Crick structures and multiple, shorter dsRNA sections can regulate PKR. To delineate the features of RNAs responsible for activation, to investigate whether p20 had any unidentified sequence specificity and to see whether a minimal RNA-activating motif could be identified, *in vitro* selection experiments were carried out here to identify RNAs that bind to the dsRBD of PKR, p20, and possibly activate PKR. A library with 10^{11} sequences was constructed and aptamers that bound to His_6-tagged p20 were isolated. Characterization revealed a novel minimal RNA motif for activation of PKR: a 16 bp dsRNA segment flanked by a 10–15 nucleotide single-stranded tail, termed as ss-dsRNA motif. Since 16 bp is the minimal p20 binding site, these data suggest that the unstructured RNA segment flanking the dsRNA binding site provides the critical determinant for activation. The ss-dsRNA motif occurs in cellular and viral RNAs, suggesting possible biological functions for PKR.

Key words dsRNA binding domain; PKR activation; SELEX; ss-dsRNA motif

Inducible Expression of siRNA in Mammalian Cells

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RNA interference (RNAi) is a powerful tool for studying gene function. To facilitate the application of RNAi to study the function of essential genes for cell growth, an inducible small interfering RNA expression system that allows a tight control of the specific gene silencing by RNAi was developed. In this system, the expression of the short hairpin RNAs is under the control of a modified human U6 small nuclear RNA promoter that contains multiple tetracycline operators and is regulated by the tetracycline-controlled transcriptional silencer Tet-tTS in a doxycycline dose-dependent manner. The tight regulation of RNAi is achieved by the strong Tet-tTS repressor that binds to the tetracycline operators in the U6 promoter in the absence of doxycycline and that completely represses the expression of short hairpin RNAs from the U6 promoter. The addition of doxycycline to the culture medium leads to the dissociation of the Tet-tTS from the Tetracycline operators in the U6 promoter, the expression of short hairpin RNAs and target gene silencing. Using this system, it is demonstrated that inducible knockdown of endogenous CXCR4 gene expression in breast cancer cells significantly inhibited cancer cell migration and invasion *in vitro*. This system should be useful for both basic research and therapeutic applications of RNAi.

Key words inducible expression; RNA interference; small interfering RNA; mammalian cell

Programming of siRNA Finding System and Its Using in Scanning and Designing of siRNA from Seven Coronavirus Genomes

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RNA interference (RNAi) has been developed as a technique for the fundamental research in life science and for drug design in the fields of gene therapy, especially in antiviral infection, such as SARS coronavirus infection. Selection of appropriate siRNA target sites was crucial for a successful induction of RNAi. A software, siRNA Finding System, was programmed by the authors using Visual Basic based on the recent RNAi researches, and some siRNA searching conditions, such as start nucleotides, GC contents and exclusion sequences in a target siRNA, could be inputted if needed. Using the software, any potential siRNA target sites in a given genome could be found rapidly and completely, and the distribution of those candidate siRNAs in any interested regions in the genome could be easily analyzed. Consensus and specific siRNAs would be given by the software when a comparison was needed between two genomes. For SARS coronavirus specific antiviral drug design, 7 coronavirus complete genomes from GenBank were scanned, and candidate siRNAs with 5' aa and with (G+C) contents between 30% and 50% were found from 1063 to 1325 for each genome. There were few consensus siRNAs between genomes. The distribution analysis indicated that more than 90 percent of the candidate siRNAs were found in 5 major genes (ORFs), that is, orf1ab, S, E, M and N. There were

454 candidate siRNAs selected according to the structural features, which related to the replication enzymes, transcription regulatory sequences, ribosome slippery sequence and sites of glycosylation or acetylation, of SARS coronavirus genome NC_004718. Among those candidate siRNAs, 446 were located in orf1ab, 2 in slippery site, 3 in glycosylation site, 2 in transcription regulatory sequence, 1 in acetylation site. This result was consistent with the principle of RNAi-based medicine design.

Key words RNAi; siRNA; bioinformatics; programming of siRNA finding; coronavirus

The Suppression of SARS Coronavirus Gene Expression by Antisense ODN and siRNA

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Antisense ODN (AS ODN) and siRNA technologies were used to suppress SARS coronavirus gene expression. The 26 sites in protein M, N and E genes of SARS coronavirus were selected as the target sites of AS ODN and siRNA. The AS ODNs were added to Vero E6 cells transfected with these genes expression vectors and the reduction effects were tested by RT-PCR after 24 hours. 13 AS ODNS could reduce target genes expression over 30%. The dose-dependent inhibition effects of 6 AS ODNs among them were then tested. The inhibition efficiencies depended on the concentrations of AS ODNs within 0–10 μM or 0–30 μM. Then, 2 siRNAs with the same sequences as 2 AS ODNs which present good inhibition efficiencies were synthesized. They were cotransfected with the target genes expression vectors to Vero E6 cells and their inhibition effects were tested by RT-PCR after 48 hours. They both had good inhibition effects. The AS ODN and siRNA technologies are proved to be good ways to cure SARS.

Targeting the 5'-Untranslated Region of Hepatitis C Virus by the Intracellularly Expressed Small Interfering RNAs

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RNA interference (RNAi) is known as a conserved biological response to double-stranded RNA, mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, and regulates the expression of protein-coding genes. It was shown that long dsRNA substrate could be cleaved into short-interfering dsRNA (siRNA), and siRNA can induce the degradation of the homologous RNA. RNAi has been cultivated as a means to manipulate gene expression experimentally and to probe gene function on a whole-genome scale.

Hepatitis C virus (HCV), a member of the Flaviviridae family of virus, is a major cause of chronic hepatitis and hepatocellular carcinoma. As an RNA virus, HCV is a prime candidate for RNAi. Indeed, it has been demonstrated recently that HCV-specific siRNA can inhibit levels of a fusion NS5B-luciferase reporter transcript when the siRNA and the target were cotransfected hydrodynamically into mice. However, as HCV is highly mutated virus, we should choose the conserved target and construct a model to choose the most effective designed siRNA swiftly and safely. Base on this consideration, we inserted the 5'-untranslated region (UTR) of Hepatitis C virus (HCV) genome into the upstream of the reporter genes of enhanced green fluorescent protein (eGFP) and luciferase, and we also constructed the expression vector that can express the short interfering RNAs（siRNA）against the HCV 5'-UTR. Then the 5'-UTR-eGFP/luciferase and the siRNA-producing plasmid were cotransfected into HeLa cells, and the inhibition effect was detected by the intensity of the fluorescence and luminescence. It showed that the light from the cotransfected cells was obviously weaker than the negative control both in quality and in quantities, and the density of the cotransfected cells had no difference with control plasmid as detected by nucleus staining. This work demonstrated that certain siRNA can target the 5' UTR of HCV, and no toxic effect had been observed in the cells. This work is the basis for future research in which RNAi activity is supposed to be utilized in the gene therapy with the HCV infection. The siRNA is expressed intracellularly by vectors instead of chemical synthesis, and a new method can be used as a model to quickly and safely screen effective siRNAs targeting HCV.

For further work, we plan to design siRNA from other parts of HCV genome and make comparison among them. Also, we will introduce the siRNA to the whole virus to see the inhibition result.

Inhibition of Hepatitis C Virus IRES Mediated Gene Expression by Short Interfering RNAs

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RNA interference (RNAi) is the process by which double-stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA in animal and plant cells. It has been found that RNAi contributes to gene function, tumor and antiviral therapy. Here we report that siRNA (short interfering RNAs) synthesized in vitro transcription by T7 RNA polymerase inhibits HCV replication by targeting HCV IRES. HCV infection was evaluated by transgenic cell model HepG2.9706. To evaluate the inhibitory activities of T7siRNA, HepG2.9706 cells were transfected using this construct via lipofectin method. Luciferase activity in cell lysates was measured for quantitative by determining antiviral effects within the cells. The results showed that the five T7siRNAs targeting to different domains of HCV IRES displayed inhibitory effects on the luciferase expression controlled by HCV IRES with the inhibitory rates of 94.31%, 80.01%, 78.01%, 80.33%, 85.64%, respectively. The highest inhibitory rate was the T7siRNA targeting the second loop of HCV IRES. It was also found that T7siRNA has a dose-dependent inhibitory effect. The inhibitory activity of T7siRNA was specific. If one base pair of T7siRNA was changed, it showed little inhibition.

Key words RNA interference; T7siRNA; luciferase; IRES; HCV

Inhibition of the Expression of Neuropathy Target Esterase in Human Neuroblastoma Cell SH-SY5Y by Antisense RNA and RNA Interference

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Neuropathy target esterase (NTE) was originally identified as the target protein for those organophosphate (OP) compounds which cause the delayed neuropathy in human and other susceptible animals, characterized by paralysis of the lower limbs and degeneration of long axons in the spinal cord and peripheral nerves. In order to explore functions of NTE by means of inhibiting its expression, two kinds of genetic vectors were constructed, which express antisense RNA and small interfering RNA (siRNA), respectively, and then transfected into human neuroblastoma cell SH-SY5Y. An antisense vector pcDNA3.1-anti-NTE was constructed by inverting subcloning an protein-coding sequence of NTE cDNA from 2324 to 3445 with specific restriction endonuclease site into pcDNA3.1(+) which was verified by DNA sequencing. To generate stable siRNA expression vector, a new genetic vector named pSUPER/neo was first constructed which can express small interfering RNA and G418 resistance in mammalian cells. To generate pSUPER/neo, *Spe*I and *Xho*I-digested inserts from pSUPER, containing H1 RNA Polymerase III promoter and the multiple cloning sites were cloned into the same sites in the pCDNA3.1(+). To generate pSUPER /neo-NTE which expresses siRNA to inhibit the expression of NTE, the pSUPER/neo vector was digested with *Bgl*II and *Hin*dIII and the annealed 64-nt oligos containing 19-nt NTE target from 430 to 448 was ligated into the vector. pCDNA3.1-anti-NTE and pSUPER/ neo-NTE were transfected into SH-SY5Y respectively by lipid-mediated method and selected with 500 g/ml G418 for two weeks, several G418-resistance foci were identified, picked and expanded. Northern blot showed the expression of NTE mRNA was inhibited in 3 SH/anti-NTE and 2 SH/ds-NTE cell clones with pCDNA3.1-anti-NTE and pSUPER /neo-NTE. NTE activity of SH/anti-NTE and SH/ds-NTE cell which express antisense RNA and siRNA respectively was 52.9±3.68 and 27.3±2.53 percent of control cell. These results will be helpful to elucidate the function of NTE.

Key words neuropathy target esterase; SH-SY5Y; antisense RNA; RNA interference

Dopamine's Role in visual Attention-like Behavior in *Drosophila*

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Background Dopamine is a major neuromodulator in both vertebrates and invertebrates, and has profound effects on many physiological processes. Many previous studies have shown that it is involved in the regulation of attention and integration of different aspects of behavior in mammals. By employing the flight simulator, selective visual attention has already been studied in *Drosophila*. Whether visual attention in flies also involves dopamine is unknown. The goal of this paper is to determine the role of dopamine in this particular invertebrate behavior. **Results** We have taken advantage of flies' orientation behavior during flight to investigate the dopamine function. Having examined several transgenic flies by the single-target orientation flight paradigm, we showed that flies with dopamine depleted from the very early developmental stage displayed a loss of orientation ability, similar to the attention loss in mammals. However, flies with transient blockade of dopamine release performed normally. Using RNA interference (RNAi) approach, we further generated flies with down-regulated J domain protein (JDP), which was a potential cochaperon in synaptic vesicle release, as another form of dopamine defective mutant. Behavioral assays revealed that JDP-depleted flies showed a similar performance defect as the dopamine mutant described above. **Conclusions** It is the effect of prolonged dopamine deprivation that influenced visual attention-like behavior of *Drosophila*.

Key words attention-like behavior; dopamine; orientation

Preliminary Researches on α1-6 Fucosyltransferase Related Mechanisms of Cancer Metastasis by RNAi

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E-cadherin is an important glycoprotein which plays a crucial role in the intercellular adhesion as well as signaling transduction. E-cadherin shows core fucosylation in high metastatic lung cancer cells while it shows loss of core fucosylation in low metastatic lung cancer cells. Since the reaction of core fucosylation is catalyzed by α-1,6 fucosyltransferase, the biological importance of core fucosylated E-cadherin is studied by RNA interference. According to the targeted sequence of catalytic domain of α1-6 fucosyltransferase, the gene-specific insert has been designed so that it specifies a 19-nt sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 19-nt sequence. The resulting transcript is predicted to fold back on itself to form a 19-base pair stem-loop structure, resembling that of *C. elegans Let-7*. Hairpin siRNA expression vector was constructed by inserting the polymerase-III H1-RNA gene promoter and the hairpin sequence in the plasmid of pBluescript. By transfecting lung cancer cells with the vector, small interfering RNAs (siRNAs) have been produced in lung cancer cells to achieve the stable suppression of the expression of α1-6 fucosyltransferase. FCM analysis and lectin blotting demonstrate that RNA interference can suppress core fucosylation of glycoproteins dramatically. Immunoprecipitation and FCM results show that core fucosylation of E-cadherin in lung cancer cells decreases apparently after RNAi. By analyzing the changes of expression levels of signaling molecules such as β-catenin and GSK3 before and after RNAi, signaling pathway of core fucosylated E-cadherin can be revealed. Therefore, the research of RNAi establishes the foundation of deep research on α-1,6 FucT related mechanisms on cancer metastasis.

Key words α-1,6 fucosyltransferase; core fucosylation; RNA interference; E-cadherin; cancer metastasis

A Novel Adenovirus Expression System for RNAi

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It's reported that some double-stranded RNA can induce the degradation of mRNA and lead to the sequence-specific gene silencing. This phenomenon is called RNA interference (RNAi). RNAi is not only an important gene regulation way *in vivo*, but also an important mechanism which can defense the invading of bacteria or virus *in vitro*. As a substitute of gene knock-out, RNAi has become a rapid and efficient method of studying gene functions in mammalian cells. Adenovirus vectors are high efficient and stable gene delivery tools in cells. Recent studies suggest that they can express small hairpin RNA (shRNA) stably in various mammalian cells. Adenovirus has two major advantages over other gene delivery systems. First, they can infect a broad range of mammalian cells including non-cycling and post-mitotic cells. Second, they can infect cells efficiently to high titers. We are developing some adenovirus-based vectors which can express RNAi-inducing shRNAs. We get a pmAd-RNAi vector including the U6 promoter and multiple cloning sites (MCS) through the homologue recombination of the transfer vector with the backbone vector. After digestion, this vector was ligated with various DNA coding sequence of shRNA, and then transformed *E. coli*. Functions of many genes can be analyzed by this directional cloning method with high-throughput simultaneously. In addition, we plan to modify the pmAd-RNAi vector for producing multiple-gene RNAi or inducible RNAi.

Key words gene silencing; RNAi; shRNA; adenovirus vector; directed cloning

SESSION 4b: OTHER RNA APPLICATIONS

Bacterial Virus Phi29 Nanomotor pRNA for Specific Delivery of Polyvalent Therapeutic Molecules to Cells

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The delivery of potential therapeutic molecules into cells has been explored for the treatment of genetic diseases, cancers, and viral infections. Gene-silencing siRNA and RNA ribozymes have shown great promise as therapeutic molecules, but their therapeutic value has been hindered by the lack of an efficient and safe *in vivo* delivery system to target specific cells. Without precise targeting, the delivery system will indiscriminately enter normal cells as well as problematic ones. The development of a safe and efficient gene delivery system that is free of pathogen-derived components is desirable. The motor pRNA complex of bacteriophage phi29 can serve as a specific targeting and polyvalent delivery system. Phi29 pRNA has a strong tendency to form hexamers, produced by the interaction of the interlocking right and left hand loops that are maneuverable and controllable. It has been shown that insertion of a ribozyme at this pRNA's 3'/5' paired ends did not interfere with the folding of either the pRNA or the ribozyme; nor with the formation of hexamers. Instead, the pRNA escorted the ribozyme to destroy hepatitis B virus. These features allow for the development of a number of new approaches. The formation of defined pRNA hexamer will allow for the construction of targeting and delivery vehicles carrying multiple components, which will include both molecules for specific cell recognition, endosome disruption and therapeutic treatment. pRNA shows unusual resistance against degradation by ribonucleases. Nucleotide derivatives, will be incorporated into the RNA to produce stable *in vitro* RNA transcripts that are resistant to RNase digestion.

Methods for Identifying RNA Accessible/Cleavable Sites and Their Application

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Anti-sense agents are valuable tools to inhibit targeted-gene expression in a sequence-specific manner and may be used for functional genomics, target validation, and therapeutic purposes. Three types of anti-RNA strategies can be distinguished: (1) use of single stranded anti-sense oligonucleotides (ASO); (2) triggering of RNA cleavage through catalytic nucleic-acids referred to as ribozymes (Rz) and DNAzymes (Dz); (3) RNA interference induced by small interfering RNA molecules (siRNA).

Target site selection constitutes the major problem in designing anti-RNA agents with optimal effect or activity, especially for long target-transcripts. Experimental approaches for identification of cleavable sites offer clear advantages. Such approaches have encompassed screening with libraries of random oligonucleotides or active Rz/Dz, or RNase-H digestion combined with an oligodeoxynucleotides (ODN) library. However, some aspects of these approaches might limit their application. We previously extended SELEX methodology to isolate guide-RNAs that can aid in the identification of accessible sites within any RNA, but it was quite labor intensive. Recently, we constructed an enriched hammerhead Rz (hRz) library to rapidly identify optimal cleavage-sites using sequencing gels; hRzs targeted to the identified sites were very active in vitro and in cell culture. Unfortunately, when Dzs were targeted to the same identified target regions, they routinely showed little or no activity, and were generally inferior to Dzs identified by the guide-RNA library. Intriguingly, one Dz identified using an enriched Dz library showed 40X higher activity than the most active Dz derived from both guide-RNA and hRz library-selections; and accessible sites identified with a modified method of ODN-library might cover all of the library-selected sites.

Our methods have demonstrated significant therapeutic potential *in vivo*. hRzs reduced Hepatitis B viral DNA and core antigen by > 80% in a transgenic mouse model, and ASOs proved extremely effective within blocking development of human papillomavirus (HPV)-induced papillomas in human foreskin grafts on immunodeficient mice. Thus far, cassettes liberating siRNAs have shown superior effects to Rzs in suppressing HPV-mRNA expression in cell culture models, and they will now be tested *in vivo* in the above mentioned models.

Key words anti-sense, accessible-site, SELEX, gene-therapy

RNA Accessible Sites Screening and Antisense Design

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Objective Gene drugs target functional gene duplex DNAs and mRNA transcripts. Considering the structure accessibility, mRNA is more accessible than the corresponding gene. Thereby antisense oligonucleotides, trans-cleaving ribozymes or RNA-cleaving DNAzymes, and siRNAs, these three basic gene silencing strategies have emerged for gene knock-down agents. The most familiar and widely used "antisense" which be introduced into a cell in the hopes that they will form Watson-Crick base pairs with the targeted mRNA, then duplexed mRNA cannot be translated, and almost certainly initiates mRNA destruction processes. But the antisense could not easily form Watson-Crick base pairs with the targeted mRNA, if only targeting the structural accessible sites. Several experimental procedures have been developed for prediction of target sites for antisense oligonucleotides binding, but the practical complexities of the methods, or poor availability of the tools, have prevented the methods from being widely used. Computational approaches have also been attempted for such prediction, but application of these prediction models beyond the training set of genes is also questionable. **Methods** A solution-based method, RNA accessible sites screening (RASS), has been developed to probe any transcribed RNA *in vitro*. The RNA was hybridized to a randomized oligo library which flanked by two fixed primer sequences. Library tags that specifically binding on the RNA molecules were collected during washing, and found precisely to define the hybridized, accessible sites of the RNA. **Results** Several gene cRNAs were screened and got the accessible sites. RNaseH cutting induced by synthesized oligos designed thereby within the probed accessible sites is more efficient than oligos of un-probed sites. *In vivo* knock-down the targeting gene has shown much more effective than the control groups. The target sites of some genes were evaluated by comparing RNAstructure3.71 based computational prediction. Screened two target sites of rabbit b-globin mRNA are the same as the two sites by combinatorial oligonucleotide arrays hybridized. RNAstructure3.71 could gave some sites which possible fell to the two sites screened by MAST, but could not figure out the exact oligos as antisense from the simulated structures. For green fluorescent protein (GFP), screened four accessible target sites were not able to selected by RNAstructure3.71. **Conclusions** RASS is a good method for designing antisense, ribozymes or DNAzymes, and helpful to develop the anti-mRNA agents.

Key words RNA; accessible site; antisense; ribozyme; DNAzyme

New Dendrimers Inhibit Group I Intron Ribozyme

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Dendrimers are molecules with highly branched and well-defined structure, globular shape, and controllable surface functionality. Their architectures consist of three parts: core, repeating unit, and end group. Dendrimers have attracted considerable interests in interactions with biological macromolecules such as DNA, proteins.

Group I intron ribozymes are self-splicing RNA molecules catalyzing the excision of themselves and the ligation of the adjacent exons. Group I introns are interesting targets for anti-microbial agents because they are present in essential genes of some microbial pathogens but not in human genome. The particular interesting group I intron-containing hosts are opportunistic fungal pathogens including *Candida albicans*, *Candida dublinesis*, *Pneumocystis carinii* that cause severe candidiasis and pneumonia. At present, treatments of those opportunistic fungal infections are very limited, calling new agents to meet the increasing clinical needs. Some clinically used drugs such as aminoglycosides and pentamidine are inhibitors of the group I intron catalysis[2]. In this study, we designed, synthesized and tested two series of polyamidoamine (PAMAM) dendrimers, with triethanolamine or modified polyethylene glycol (PEG) as the core and the function of ester and amine as the end group respectively. Our results showed that the dendrimers with primary amines as the end group could remarkably inhibit the activity of a group I ribozyme from the 26S rRNA gene of *C. albicans*, while those with ester group at the end had no inhibition. The inhibition potency was increased with increasing generation of the dendrimers, where increasing numbers of primary amines were present at the surface. The most potent dendrimer found in our study was the dendrimer with triethanolamine as the core and 48 amines at its surface, having a value of IC_{50} around 400 nM. The strong inhibition of ribozyme induced by our synthesized dendrimers can be explained by the electrostatic interactions and the hydrogen bonding. The *in vivo* activity of these dendrimers in inhibition of the group I intron splicing and the growth of the host pathogenic fungi needs to be tested.

SESSION 5: rRNA & tRNA

Mapping and Comparative Study of 2'-O-Ribose Methylated Nucleotides in Rice 18S rRNA

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Eukaryotic rRNAs contain a large number of 2'-O-ribose methylated nucleotides of elusive function, which are confined to the universally conserved rRNA domains. Although early biochemical data showed that higher plant rRNAs contain many 2'-O-ribose methylated nucleotides, until very recently only the 2'-O-ribose methylation sites in rice 25S rRNA have been investigated. Here, we report a systematical mapping of such sites in rice 18S rRNA.

A series of primers were designed for reverse transcription of rice 18S rRNA and 2'-O-ribose methylation sites were revealed by the pauses of primer extension at lower concentration of dNTPs. In this study, 35 2'-O-ribose methylated sites were precisely mapped, among which 31 had been predicated by the rice box C/D snoRNAs identified previously from our laboratory. The remaining 4 sites are newly determined in rice 18S rRNA and their corresponding snoRNAs have not been found yet. Compared with the known methylation sites of human and yeast 18S rRNA, ten of the 35 sites are conserved between rice and both of the two organisms while 9 residues are conserved in either human or yeast rRNA. Moreover, comparing the 35 nucleotides with the known methylation sites in *Arabidopsis* rRNAs, 25 are conserved between two plants, including 7 that are plant specific. Interestingly, nine of the remaining ten methylated nucleotides appear unique to rice 18S rRNA.

It is shown by this study that many methylation sites in rRNAs are conserved among rice and other eukaryotes, especially between rice and *Arabidopsis*. However, a relatively high proportion of the methylation sites appear rice or monocotyledonous plant specific. This result demonstrated the diversification of 2'-O-ribose methyl sites even in the two flowering plants despite the high similarity in their rRNA sequences, implying a more complicated rRNA methylation pattern in plants than had been previously thought.

Key words 2'-O-ribose methylation; rRNA; snoRNA; rice

A Comparative Study on Toxicity of Two Type II Rips (Cinnamomin and Ricin) to Silkworm Larvae at Both Cellular and Molecular Level

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Cinnamomin and ricin are two type II RIPs. They exhibited different toxicity to silkworm (*Bombyx mori*) larvae by oral feeding bioassay. The IC_{50} of ricin to the silkworm larvae at third instar was much lower than that of cinnamomin. When the isolated 80S ribosome from silkworm pupae was treated separately with the reduced cinnamomin or the reduced ricin, a specific RNA fragment (R-fragment) was produced as characterized by 8 M urea-denatured polyacrylamide gel electrophoresis. The purified A-chains of both cinnamomin and ricin showed approximately identical RNA N-glycosidase activity to the silkworm ribosome. It was proposed that the difference of their toxicity to silkworm larvae was not because of their A-chains, but of the properties of their B-chains. It was also found that the liquid extracted from the midgut of silkworm larvae could hydrolyze these two proteins apparently to the same extent.

Key words cinnamomin; cytotoxicity; ricin; RNA-glycosidase; sarcin/ricin domain; silkworm

Both N- and C-Terminal Regions are Essential for Cinnamomin A–Chain to Deadenylate Ribosomal RNA and Supercoiled Double-stranded DNA

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Cinnamomin is a type II ribosome-inactivating protein and its A-chain exhibits RNA N-glycosidase activity to remove an adenine in the conserved sarcin/ricin loop of the largest RNA in ribosome, arresting protein synthesis at the elongation step. In this report, deadenylation of both ribosomal RNA and supercoiled DNA by native and recombinant cinnamomin A-chain expressed in *E. coli* was demonstrated. However, the mutants of cinnamomin A-chain devoid of N-terminal 52 or/and C-terminal 51 amino acid residues lost activities of both RNA N-glycosidase and to release adenines from supercoiled DNA. Additionally, supercoiled DNA could not be cleaved into nicked and linear forms by these mutants. These results indicate that both N- and C-terminal regions are essential for activity of cinnamomin A-chain to deadenylate ribosomal RNA and supercoiled DNA. It was suggested that phosphodiester bonds in the extensively deadenylated region of supercoiled DNA would become fragile and liable to be broken spontaneously owing to the existence of tension in the supercoiled DNA.

Key words cinnamomin A-chain; deadenylation; deletion mutation; ribosome-inactivating protein; supercoiled DNA

Specific Cleavage of the Phosphodiester Bond between C4453-A4454 of Rat Ribosomal 28S RNA by Orientin Resulting in Inactivation of Ribosome

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A novel ribosome-inactivating protein (RIP) named orientin was purified from mature seeds of oriental arborvitae (*Biota orientalis*). Orientin is a RIP of ribonuclease type (MW is about 13 kD). When the concentration of magnesium was 25 mM in the incubation buffer, orientin specifically hydrolyzes the phosphodiester bond between C4453 and A4454 in region K (a region in domain VII) of rat 28S rRNA, thus causing inactivation of ribosome. The region around C4453-A4454 in 28S rRNA is named as orientin region. The orientin-treated ribosome produces a small RNA fragment (S-fragment) that contains 333 nucleotides from the 3'-terminus of rat 28S rRNA. The distance is 128 nucleotides between the cleavage-sites of á-sarcin (G4325) and orientin (C4453). Under the restricted condition (25 mM magnesium), the substrate specificity of orientin is extremely high: it acts only on the orientin region of the largest RNA in ribosomes from certain eukaryotes. The specifically damaged ribosome caused by orientin affects EF-1a-dependent binding of aminoacyl-tRNA, whereas it does not affect the formation of the EF-2/GDP/ribosome complex. It is proposed that orientin inactivates ribosome at least partially by interfering with the

EF-1a-dependent binding of aminoacyl-tRNA to ribosome in the elongation step of protein synthesis. Orientin might be used as a powerful tool in studying the structure and function of ribosome in protein synthesis

The Polarity of E153 in *B. subtilis* TrpRS is Essential for the Activation of Tryptophan

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From residue P^{143} to A^{155} in *B. subtilis* TrpRS is a very conserved domain located between "GXDQ" and "KMSKS" in the tryptophanyl-tRNA synthetases. These 13 amino acids belong to the catalytic domain that is in charge for the activation of tryptophan and the aminoacylation of $tRNA^{Trp}$. Based on the recent data of the TrpRS-TAM complex crystal structure, the position of residues and the interaction between the residues and tryptophan or ATP have been illustrated in detail. E^{153} locates in the binding pocket of tryptophan. It provides an H-bond to or an electrostatic interaction with the substrate-tryptophan. To study the importance of glutamate, E^{153} was mutated to D^{153}, K^{153} and G^{153}, respectively. The kinetics parameters of three mutant TrpRSs were determined. There were no significant changes in the values of Km and k_{cat} when E^{153} was mutated to D^{153}. After E^{153} became to K^{153}, both the Km and the k_{cat} increased near 30-fold. But the relative activity of the second mutant did not change so much compared with the wild-type TrpRS. Once E^{153} was changed to G^{153}, the reaction of tryptophan activation seemed not to occur even as the concentration of TrpRS had been added to 10,000-fold of the other usage. The CD spectra of the wild-type TrpRS and three mutants were similar; the mutagenesis had little effect on the secondary structure of the enzymes. Both the kinetics data and structural parameters support that the polarity of E^{153} is important to the processing of tryptophan activation.

Key words TrpRS; polarity of E^{153}; activation of tryptophan

The C-Terminal of *Bacillus subtilis* Tryptophanyl-tRNA Synthetase

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To study the function-structure relationship of *Bacillus subtilis* tryptophanyl-tRNA synthetase, a series of mutations and deletions were constructed focusing on its C-terminus. After expression and purification of these mutations, their kinetics constants for both ATP-PP exchange and aminoacylation were determined. The results show that residue R327 is important to this enzyme. The potential role it might play in inter-domain communication was discussed.

SESSION 6: VIRAL RNA & ANTI-VIRAL THERAPEUTICS

A New Method for Denaturing dsRNA and Developing BTV Molecular Detection Kit

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Bluetongue virus (BTV) is an arthropod-borne Orbivirus that causes the infective bluetongue disease in wild and domestic ruminants. In all over the world, 25 serotypes of BTV have been found. This is a non-enveloped virus containing 10 molecules of double-stranded RNA (dsRNA) as its genome. Segment 7 is a gene deciding its serogroup-specific antigen VP7. Among all serotypes, most amino acids (>94%) of VP7 including the location (position 255) of a single lysine residue are conserved. Hitherto, there is still not any effective way to prevent and therapy this disease. In our previous studies, it has been concluded that this BTV cannot infect any of the normal human cells, has no capability to induce the normal cell into the cancer cell. Why dose this virus have so selective characteristic of biology? We are studying the feature of BTV dsRNA.

Our work also showed that there are many inconveniences in studying dsRNA, for example, the denaturing of the dsRNA in the preparation of positive or negative strand RNA. It was found in our recent work that severe longer time under high temperature combined with the salt is necessary for the denaturing of the dsRNA. In order to demonstrate that this method is simple, efficacious and stable for denaturing the dsRNA, we applied it in the denaturing of the dsRNA to develop the BTV molecular detecting kit by RT-PCR technique combined with cloning and sequencing of the PCR products.

A pair of group-specific primers corresponding to the conservative sequence of S7 fragment of BTV genome and the reverse transcription polymerase chain reaction (RT-PCR) technique were used to amplify the 5′NCR region of segment S7 from the two sains of BTV (BTV-HbC, BTV-10). Then two product sequences were cloned into two pUCm-T vectors respectively by ligating of cohesive ends. The accuracy of these two recombinant plasmids were checked by using PCR, restriction enzyme analysis, and sequencing (No. ABI PRISM377). Efficiency of the test method was evaluated with determining the diagnosis veracity to disease and experiment specimen of animal.

The results indicated that this method is very excellent to be used in the denaturing of the dsRNA; S75′NCR sequence of BTV-HbC is 277 bp, and BTV-10 290 bp. Our RT-PCR system is a rapid, sensitive and precise system for examining the infection with the bluetongue virus.

Key words double-stranded RNA (dsRNA); dsRNA virus; the denaturing of the dsRNA; RT-PCR of the dsRNA

Purification of dsRNA Bluetongue Virus HbC with a New Immunoprecipitation Method

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Because of its special dsRNA genome and complicated serotypes, bluetongue virus (BTV) is a better study material for the molecular structure and function, heredity and variation of organism, as well as the interactions between virus and cells.

Bluetongue virus HbC strain (BTV-HbC) is a new BTV strain isolated from Hubei Province of China by our laboratory in 1987. During the research on BTV-HbC, we found that this BTV-HbC is different from other BTV because it could kill some human cancer cells. So it is necessary to approach to a simple and efficacious method to acquire a great lot of high pure bluetongue virus particles for researching the structure and function of it, interaction between BTV-HbC and some cancer cells, etc..

A new immunoprecipitation method introduced in this article is quite different to classical ones for it's reverse mode of thinking. The cellular material in the virus suspension extracted from cell culture was immunoprecipitated using an anti-cellular antibody combined with appropriate concentration of some salts. The BTV-HbC particles could be separated from cellular material and the effective purification method has been developed for virus particles of bluetongue virus HbC. The purified particles were analyzed by PAGE, electron microscopy, gel chromatography and tissue culture for checking up the purification degree and infectivity of BTV-HbC.

Key words bluetongue virus HbC; purification of BTV-HbC; immunoprecipitation; oncolytic virus; gene therapy

The Methodological Study of Detecting HCV RNA by Combining RT-PCR with Transcription *in Vitro*

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Objective To establish a new method that is based on transcription *in vitro* combined with a single step reverse transcription PCR, in which a great lot of ssRNAs can be synthesized specifically and HCV RNA can be detected sensitively. **Methods** The serum of the patients who had been infected with hepatitis C virus were used as specimens, and HCV RNA was extracted from these specimens by the commercial extract kit (Trizol). Single step reverse transcription-PCR (SRT-PCR) was performed prospectively with a set of primers that amplified a 315 bp sequence unique to the 5′noncoding (NC) region of the HCV genome. Then, the transcription *in vitro* was performed with another designed pair of primers, one of which contained a binding site of bacteriophage T7 RNA polymerase. In the transcription *in vitro*, the RT-PCR products were acted as the templates. As a result, in the presence of T7 RNA polymerase and other appropriate conditions, a great deal of specific single strand RNAs were synthesized even in one-hour. Then these products were examined for identifying the method specificity and sensitivity by the electrophoresis on agarose gel and the dot hybridization with the specific probe. **Results** The amplification rates of this transcription *in vitro* combined with RT-PCR between SRT-PCR and the standard RT-nested PCR was similar. Additionally, the length of the transcription products accords with expected size by the electrophoresis on 2% agarose gel containing ethidium brimide, while the products' specificity was verified by the dot hybridization with the specific probe. We also detected the nucleic acids of HAV, HBV, HEV, HGV, CMV and EBV by the same method. It turns out that no visual band except that which was amplified from HCV specimen can be found. These results demonstrated that this method we developed was specific. Furthermore, as we amplified a series of dilution serum by this method, the outcome showed that this assay would be able to detect HCV at or beyond the sensitivity level of one chimpanzee infectious dose (CID). **Conclusions** This assay strategy has the characteristic of simplicity, rapidity and specificity, as well as sensitivity, which can be used as a HCV RNA detecting method, especially to low HCV concentration. In addition, this method can be used as a powerful tool of molecular biology to synthesize functional mRNA or antisense RNA, to produce labeled single strand RNA probe, and even to sequence PCR products directly and rapidly. Moreover, for the final product of this assay is the specific single strand RNAs, it is predictable that, with simple annealing procedure, this method is useful even to the newly discovered phenomenon—RNA interference (RNAi) too.

Key words transcription of RNA *in vitro*; synthesis of RNA *in vitro*; Hepatitis C virus

Primary Investigating of BTV-HbC Targeting and Infecting Some Human Cancer Cell Lines

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Bluetongue virus (BTV) is a member of the genus *orbivirus* belonging to *Reoviridae*, and 25 serotypes have been found all over the world. This virus can cause animal Bluetongue disease on sheep and wild ruminant, and cattles are its reservoir host. But it can't cause any disease of human. Complete BTV virion has not envelope, and its genome consists of 10 molecules of double-stranded RNA (dsRNA), and each molecule of dsRNA codes for one of structure or function protein polypeptides.

BTV-HbC was isolated from Hubei Province of China by our laboratory in 1987. Fairly systemic studies on its biological and molecular biological characteristics have been made. According to the analysis of its gene map, it was probably supposed to be a new genotype. On the basis of comparing with the international standard strains, this study further investigated the biological characteristics of BTV-HbC strain in various species monolayer cells and the ultrastructural change characteristics of cells infected with it. Furthermore, through the serological cross-immunologic reactions with BTV-10 strain, we further identified the serological feature of BTV-HbC.

Some researchers have suggested that BTV can not exist and duplicate in human body normal cells and has no destroy to human tissue cell. But in the research on Bluetongue Virus HbC strain, we found that BTV-HbC could kill some of human cancer cells such as Human lung cancer cells-SPC-A1, Human cervical cancer cell HeLa, human star Glioma cells U251, human liver cancer cells 7402, human liver cancer cell Hep-3B, and in a sharp contrast against the normal human embryo lung cells (HEL). Cytopathological effect (CPE), analysis of cellular ultrastructure changes with transmission electron microscopy (TEM) and RT-PCR of BTV-HbC genetic sequences were used to detect the status of all culture cells infected with BTV-HbC. The results show us that the BTV-HbC could infect and kill some human cancer cells maybe with

targeting.

Key words dsRNA Bluetongue virus; interaction between BTV and human cancer cells; Bluetongue virus HbC strain

Generation of Multiple mRNA Transcripts from the Novel Human Apoptosis-inducing Gene Hap by Alternative Polyadenylation Utilization and the Translational Activation Function of 3' Untranslated Region

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hap, a novel human apoptosis-inducing gene, was identified to have two major mRNA species of 1.8 and 2.7 kb in length by Northern blot analysis of poly(A)(+) RNA from multiple human tissues. Rapid amplification of cDNA 3′-end (3′-RACE) was used to get 3'-end of *hap* gene, then its products were verified by southern blotting and sequencing. The results prove utilization of alternative polyadenylation signal generates multiple mRNA transcripts from the apoptosis-inducing gene *hap*. The two hap transcripts derive from the alternative polyadenylation site selection: an AATAAA signal at position 1528–1533 nt for the 1.8 kb mRNA, and an AATAAA signal at position 2375–2380 nt for the 2.7 kb mRNA. The 3′-UTR spanning the region between the second and the third polyadenylation site of 2.7 kb Hap was demonstrated to exert a translational activation function for *hap* itself and the chloramphenicol acetyltransferase (CAT) reporter gene expression by approximately three-fold, despite no differences observed in the steady-state level of relative cytoplasmic mRNA. Comparing the mRNA stability of two hap transcripts indicated that the longer mRNA was not more stable than the short one. Taken together, all these data provide evidence that the hap 3′-UTR containing within the second and the third polyadenylation signal can regulate gene translation rather than transcription and mRNA stability.

The 321 Residues in the C-Terminal of Spike Proteins (SARS-CoV) Can Be Used as Diagnostic Antigen for SARS Virus Infection

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In March 2003, a novel coronavirus has been identified as the causative agent of severe acute respiratory syndrome (SARS). The SARS-associated coronavirus has been named as "SARS virus" (SARS-CoV) and is a positive-stranded RNA viruses. It is also an enveloped viruse that deliver RNA genome into the host cells by binging to the cellular receptors and subsequent coalescence of the membranes surrounding virus and cell. In these events the S proteins are responsible for both receptors binding and membrane fusion. Within the trans-Golgi network, the S protein is cleaved into two non-covalently bound subunits by host cell-derived protease, an N-terminal half peripheral S1 and COOH-terminal half membrane anchored S2. S2 contains the core machinery necessary for membrane fusion, so it may contain a part of main epitopes of S protein. Here we report that the 321 residues in the C-terminus of S2 (S2-321) can specially detect and bind to the antibody in the sera of different SARS patients from Guangdong and Hubei province in China, that significantly present a prospect for rapid and special SARS detection. We cloned S2-321 cDNA into the pET-28 vector which was used to transform *E. coli* BL21(DE3). After induction with isopropyl-1-thio-*D*-galactopyranoside, S321 was proved to be expressed by SDS-PAGE and Western blot. Special anti-SARS-CoV antibodies were tested in a direct binding ELISA in a 96-well microtiter plate coated with purified S2-321 mentioned above. Among the total 36 samples of sera from probable (21 cases), suspected SARS patients (cases) and hospital's healthcare workers, 21 probable cases were unexceptionally tested as positive. Moreover, combined with SARS detection kits produced from HUADA Co and Hongkong respectively, 5 suspected cases were also tested as diagnostic positive. Our work endeavoring in the S2 functional regions initially paved a way to this approach and may add a hand to international collaboration of preventing this global pandemic of SARS.

Novel Subgenomic RNAs and Noncanonical Transcription Initiation Signal of Severe Acute Respiratory Syndrome Associated Coronavirus (SARS-CoV)

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Severe acute respiratory syndrome (SARS) is a febrile respiratory illness. The disease has been etiologically linked to a novel coronavirus that has been named the SARS-associated coronavirus (SARS-CoV), with positive strand RNA genome of approximately 29.7 kb. Since it is a member of the Coronaviridae, a complex transcriptional, translational and posttranslational regulatory mechanisms is involved in SARS-CoV genome expression. The expressions of the genetic information of SARS-CoV involve synthesis of a set of successively smaller subgenomic mRNAs, which are responsible for synthesis of different viral structural and non-structural proteins. The exact number of SARS-CoV subgenomic mRNAs and molecular mechanism underlying the synthesis of SARS subgenomic RNA is still not clear. In order to address these questions, total RNA from SARS-CoV infected Vero cells was extracted and subjected to Northern blot analysis with probes specific for 3' non-translated region. At present, exact number of subgenomic mRNAs, existence of their corresponding negative strand subgenomic RNAs and precise leader to body fusion sites were determined by RT-PCR and DNA sequencing.

We characterized 10 subgenomic mRNAs corresponding to ORFs S, S′, X1, X2, E, M, X3, X4, X6 and N in SARS-CoV infected Vero cells. We are the first to report the existence of two previously unrecognized novel subgenomic mRNAs termed as S′ and X2. Sequence analysis revealed leader body fusion site of the mRNA S′ is located 384 nucleotide downstream of the consensus Trans Regulatory Sequence (TRS) for the spike protein mRNA. Body TRS of the S′ has only 4 nucleotide homology with consensus TRS of SARS-CoV. ORF of S′ subgenomic mRNA is predicted to encode a 124.2 kD truncated spike protein. Leader to body fusion site of X2 subgenomic mRNA is located 13 nucleotide