The potential immunological functions of pseudogenes and other 'junk' DNA

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The distinction between functional gene copies and supposedly non-functional ones (pseudogenes) is becoming ever harder to determine. The unexpected discovery of a human retropseudogene that codes for a tumor antigen recognized by T cells follows earlier discoveries of protein-encoding genes that exhibit the secondary capability of producing short antigenic peptide segments from alternative reading frames. Tumor antigens themselves enjoy mixed success in enabling the organism to destroy tumor cells, and one major research objective is to increase their effectiveness for the creation of therapeutic anti-cancer vaccines.

A function for noncoding DNA itself is suggested in terms of an organism's antiviral immunological strategy. Noncoding DNA is capable of being widely transcribed, and such transcripts can lead to the formation of strands of double stranded RNA (dsRNA). The latter is capable of inhibiting overall protein synthesis (notably that of viruses) once its formation is triggered by a viral infection. If correct, then this also accounts for the profusion of noncoding DNA in the human genome. The apparent profligacy of junk DNA is necessary to endow it with sufficient polymorphism to make it likely to lead to the origin of dsRNA complexes with whatever 'nonself' viral transcript comes along. This, in effect, makes it difficult for a virus to anticipate the host's dsRNA-based defensive response.

At one time, the distinction between a functional and non-functional gene copy seemed virtually self-evident. The disabled state of certain gene copies (pseudogenes) was summarily reckoned from 1) the inferred absence, or mutational alteration of, the promoter, 2) the apparent absence of a suitably situated initiator codon, 3) an open reading frame (ORF) containing several inferred missense mutations, and/or 4) an ORF interrupted by one or more frameshifts and/or premature stop codons. As demonstrated elsewhere,[1] *none* of these seeming disablements can any longer be trusted as conclusive indicators of a pseudogene's inert status. Furthermore, there are two snail pseudogenes that are functional in spite of containing such seeming disablements,[2] and such is also the case for a recently described murine pseudogene.[3] This, of course, does not include the cases where so-called pseudogenes are simply relabelled as genes upon the discovery of function, or genes that contain pseudogenic features that are circumvented by genomic recoding processes.[1]

Now comes further evidence of unconventional gene/pseudogene behaviour. Many protein-coding genes, in addition to their long-known role of directing the synthesis of peptides from their conventional open reading frames, also produce short peptide segments from partial or unconventional open reading frames.[4] These short peptides often have auxiliary functions relative to the product synthesized from the main open reading frame. In addition, other short peptides are capable of serving as tumor-rejection antigens. A growing list of such antigenic peptides is available online.[5]

Even more surprising is the fact that a human pseudogene, *NA88-A*, conventionally deemed incapable of producing anything even resembling a biologically meaningful peptide, has also been found to produce a tumor rejection antigen from an alternative open reading frame. It is the main subject of this report. This and related discoveries open up a whole world of previously unsuspected potential immunological functions for various types of junk DNA.

A synopsis of T cell function

In order to help the reader understand the potential significance of the antigen-producing *NA-88A* pseudogene, a brief summary of the relevant part of the immune system is now presented. For more comprehensive information, the reader is referred to two excellent introductions to this subject.[6,7] Otherwise, the overall process by which T cells can destroy tumor cells (Figure 1) is described in some detail below during discussion of tumor antigens.

Although there are means by which the host immune system can combat a viral infection without destroying infected cells,[8] attention is focused on the processes that lead to such destruction. The lymphatic system is responsible for the transport of specialized cells (lymphocytes) whose function is to destroy foreign elements in the body. One type of lymphocyte, having originated in the bone marrow, matures in the thymus gland (an organ once thought to be vestigial) and is hence called the T cell. Lymphocytes normally do not enter individual infected cells (except for vesicles) in search of the 'non-self' entity that has taken residence within an individual cell. Instead, they identify the infected cell, attach to it, and destroy it entirely (Figure 1). It is for this reason that they are referred to as killer T cells or cytolytic T cells (CTLs). The successful destruction of a 'nonself'-infested cell, if done in a timely manner,

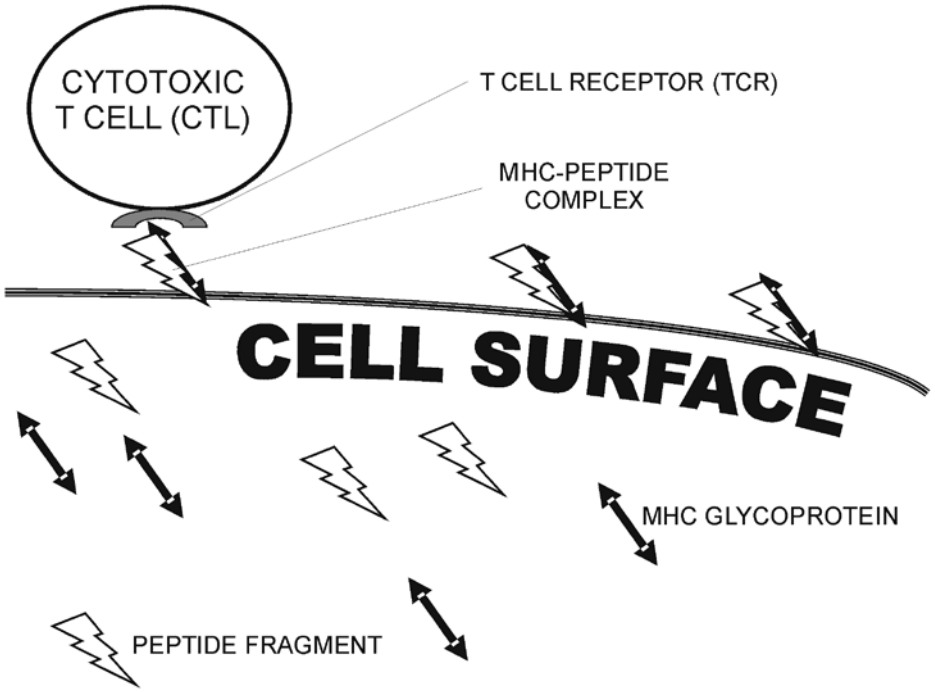

Figure 1. *Peptide fragments from within the cell, regardless of origin, form complexes with the specialized MHC glycoproteins synthesized within the cell. These MHC-peptide ligands are then presented at the cell surface. If a cytolytic T cell (CTL) senses that some of the peptides thus presented are of a 'nonself' nature, its receptor (TCR) docks to the MHC-peptide ligand, and, assuming that other conditions are met, proceeds to destroy the affected cell.*

usually limits the spread of an infection.

For T cells to be able to monitor intracellular content, samples of the entire cell interior must be continuously presented at the cell's surface. This takes place in the form of short peptide fragments (usually 8–11 amino acids in length), numbering perhaps 10,000 per human cell,[9] each of which becomes complexed with a special Major Histocompatibility Complex (MHC) molecule, which then travels with the bound peptide fragment to the cell surface. It is only then that the T cell is able to discern the potential presence of complexed peptide fragments that have originated from pathological processes within the cell:

> 'Adaptive immunity provides the basis of recognition of foreign pathogens and tumors [Refs.]. This specific immunity is dependent on the recognition function of the αβ T cell receptor (TCR) on T lymphocytes that detects a protein fragment (i.e. peptide) of a self-protein or cell-associated pathogen (derived from either viral, bacterial, fungal, parasitic or tumor cell origin) bound to an MHC molecule.'[10]

The TCR will connect with the 'nonself' peptide-MHC ligand, commencing a series of processes by which the T cell will destroy this cell. In contrast, T cells will not connect to or destroy any cells displaying only 'self' peptide-MHC ligands at the cell surface, unless of course the organism has an autoimmune disorder.

Antigenic peptides produced by alternative open reading frames

Traditionally, the intracellular peptide segments which serve as antigens (zigzags in Fig. 1) have been thought of as having originated from the breakdown products of used-up functional proteins within the cell. We now realize that, in addition, incorrectly-synthesized proteins are broken down within the cell almost as rapidly as they form, and short segments of these malformed peptides are also presented on the cell surface in the form of MHC-peptide ligands (arrow-zigzag unions, Fig. 1). Functional proteins are usually employed within a cell for an appreciable period of time before being broken down and presented at the cell's surface. By contrast, malformed proteins are presented at the cell's surface very soon after being synthesized and then broken down. A virus invading a cell betrays its presence as soon as it produces 'nonself' malformed proteins. Any functional viral proteins synthesized and used within the cell would not appear as eventual breakdown products on the cell surface until much later. The inclusion of the remnants of recently synthesized protein segments, as part of the immunologic repertoire, is believed to facilitate the quick response of CTLs to fast-acting viral infections.[11] Otherwise, viruses would get too much of a head start, in terms of their proliferation, were the immune system to have to wait for the belated appearance of fragments belonging to used-up functional viral peptides before being triggered into action.

A second major departure from the exclusive employment of previously used functional proteins as antigens is the use of short peptide segments produced by the alternative open reading frames of genes.[12] This is, in effect, a *secondary* synthesis of 'ready-made' short peptide segments for the apparent sole purpose of provoking a T cell response. Mayrand and Green[13] have described a few examples, and several more have been discovered since. They assess the immunological significance of this unconventional gene behaviour as follows:

> 'Infusion of TIL586 and interleukin 2 (IL-2) into the autologous patient resulted in regression of metastases, demonstrating that alternative ORF-derived peptides can have *in vivo* immunological importance… . Regardless of the mechanism(s) of

generation, the existence of previously undefined, and therefore unconsidered, pools of antigenic peptides requires attention with regard to their role in immune system development and function.'[14]

It has generally been supposed that the alternative open reading frame of a gene can lead to the synthesis of an antigenic peptide only while the main open reading frame is active in the synthesis of the main gene product. However, we now realize, as exemplified by the M-CSF gene,[15] that an alternative open reading frame can be translated in tissues in which the main open reading frame is not expressed at all. The ability of an alternative open reading frame to become activated independently of the behaviour of the main open reading frame raises gene behaviour to a new level of complexity. It also helps us understand how a pseudogene can possess a functional alternative open reading frame despite the (actual or supposed) fact that the main open reading frame is inactive.

The unexpected discovery of a tumor-antigen producing pseudogene

In contrast to genes, pseudogenes are usually reckoned as having disabled open reading frames. However, regardless of the correctness or otherwise of this supposition, researchers increasingly realize that pseudogenes can possess functional *segments* of the original open reading frame.[1] This constitutes a form of alternative open reading frame usage.

The human *NA88-A* pseudogene is conventionally believed to be a defective copy of the *HYX42B* gene. The latter codes for a homeoprotein, is located on chromosome 10, and is transcribed in a variety of normal tissues. The *NA88-A* pseudogene is, based on its sequence relative to the paralogous *HYX42B* gene, believed to be incapable of directing the synthesis of a homeoprotein. This does not, contrary to the usual way of thinking about pseudogenes, necessarily mean that the *NA88-A* pseudogene lacks function. Unlike the *HYX42B* gene, the *NA88-A* pseudogene codes for an antigenic peptide segment from a short open reading frame.[16]

$CD8^+$ T cells recognize this antigen on melanoma cells. The $CD8^+$ T cell clone exhibits no lytic activity, but is capable of secreting TNF (tumor necrosis factor) and IL-2 (interleukin 2) when stimulated by melanoma cells.[19] (Note that *in vitro* $CD8^+$ T cells have been shown to have far greater antitumor potency than TILs (tumor-infiltration lymphocytes).[17] Furthermore, IL-2, a product of activated $CD8^+$ T cells, is known to drive the further proliferation and differentiation of these cells, leading to the emergence of armed effector T cells, which can then attack and destroy infected cells[18]).

The antigenic expression of *NA88-A* pointedly warns against assuming that even a pseudogene with a highly 'mangled' open reading frame (using an indisputably-functional paralogous protein encoding gene for reference) is necessarily 'dead':

'The *NA88-A* gene exhibits several premature stop codons, deletions, and insertions relative to the *HPX42B* gene. In *NA88-A* RNA, a short open reading frame codes for the peptide MTQGQHFLQKV [letters are standard abbreviations for specific amino acids] from which antigenic peptides are derived; a stop codon follows the peptide's COOH-terminal Val codon.'[19]

In contrast to the pseudogene, the paralagous protein-coding gene, *HPX42B*, is not capable of appreciable T cell stimulation.[20] Ironically, one of the premature stop codons in *NA88-A*, usually considered one of the most obvious of 'gene killers' in a pseudogene, actually plays an essential role in the production of this antigen. Experimental alteration of this stop codon abolishes production of the antigenic peptide.[21]

The discovery of the *NA88-A* pseudogene has facilitated, if not compelled, the re-examination of pseudogenes in a more favourable light. For instance, in their study of the Ψ*mtTFA* pseudogene, Mezzina *et al.*[22] suggest that Ψ*mtTFA* lacks function based upon the truncation and inferred mutational scrambling of its open reading frame, but nevertheless raise the following caution:

'… we cannot rigorously exclude that the Ψ*mtTFA* might have evolved to fulfill some alternative function, in which case, the sequence drift could be ascribed to selection for this alternative function rather to a lack of functionality. This is the situation described for the homeoprotein HPX42B, where the pseudogene codes for an antigen that is recognized by the CD8(+) T cells while the expression of the gene does not lead to antigen production.'[16]

Moreover, *NA88-A* expression has broad implications that include, but are not limited to, the possibility that large numbers of pseudogenes are at least potentially functional:

'The *NA88-A* antigen is encoded by a very short ORF, which was derived from part of a functional mRNA's 3' UTR [Untranslated Region]. Our results thus extend the possible sources of tumor antigen coding sequences to 'junk' DNA and seriously raise the possibility that any DNA sequence can lead to antigen production, the only limitation being that it must be transcribed.'[21]

The immunological versatility of seemingly scrambled and therefore supposedly useless pseudogene sequences is clear. An additional factor relevant to this versatility stems from the previously known fact that potentially antigenic peptide fragments that bind to MHC molecules (including Class I molecules) do not themselves require a precise amino acid sequence to bind to these molecules for presentation on the cell's surface for CTL 'examination' (Figure 1). To the contrary, point connections, based upon relatively weak hydrogen bonds or ionic interactions[23]

and dependent upon a few conserved amino acids known as anchor residues, are sufficient for effective binding of MHC-peptide ligands.

However, the foregoing discussion does not imply that *any* random short peptide fragment would necessarily be capable of forming a complex with some MHC variant. To begin with, antigenic activity from the cDNA of this pseudogene is limited to the final 700–732 segment, and is completely absent in 1–700, and this activity is further limited to the correct open reading frame.[20] Note that, in addition to the previously discussed strategic position of the premature stop codon, only a small 8–11 amino acid portion of the *NA88-A* pseudogene sequence stimulates T cell activity. Moreover, experimental alteration of the antigenic peptide itself, MTQGQHFLQKV, shows that alterations at the front end (the addition of 1 amino acid or the removal of either 1 or 2 amino acids), has little effect on the antigenic properties of this peptide segment. The same cannot be said about removal of the terminal valine (V) residue.[20]

Characterizing the tumor-antigen producing pseudogene

There are several types of tumor antigens known.[5] A few of these are the ultimate result of mutations induced by the tumors themselves, but not in the present instance:

> 'In conclusion, the *NA88-A* gene codes for a melanoma-specific antigen today, in large part because, during its evolution, a point mutation transformed a Trp codon into a stop codon. This significantly augmented production of antigenic peptide.'[20]

Another set of antigens express themselves in immunologically privileged sites such as the testis, but in no other healthy tissue. In fact, the *NA88-A* pseudogene is expressed not only in tumors but also in testis[24] (itself indicative of a potential function in testis), meaning that it constitutes a tumor non-mutated self-protein. If *NA88-A* expression is normally limited to testis, then the very appearance of the *NA88-A* in non-testicular tumors itself serves as a 'nonself' alarm signal. (Note that, in other contexts, the so-termed cancer/testis antigens are regarded as attractive targets for immunotherapy[25]).

There are other possibilities, including the oncofetal antigens, the sole expression of which in healthy tissues occurs during early development.[26] Still other tumor antigens express themselves to some extent in a variety of normal tissues. So which type of tumor antigen is the one produced by *NA88-A*? Unfortunately, this pseudogene has not been tested for expression in a variety of normal, healthy tissues. If, however, *NA88-A* turns out to be expressed in several healthy tissues, then its role as an immunological 'alarm bell' for the presence of the 'nonself' tumor probably stems from a timing and/or abundance of expression that differs from that of healthy tissue.

There have not, to this author's knowledge, been any further studies conducted on the *NA88-A* pseudogene and any immunological function it may perform. One specialist with whom I have discussed this pseudogene has suggested that *NA88-A* may be actively destroying tumor cells, but one would never know this because of the fact that the successful execution of this function destroys the very evidence of its success. Owing to our limited understanding of the *NA88-A* pseudogene itself, a more broad-based approach to the understanding of its potential significance is necessary. The attention of this article is now turned to tumor antigens in general, especially those that, like *NA88-A*, originate from the expression of alternative ORFs.

Assessing the immunological significance of tumor antigens

Tumors, by virtue of their pathological nature, are a type of 'wild' tissue, and it is not surprising that they can activate genes and pseudogenes from an alternative ORF that may never be otherwise expressed. However, it is by this very behaviour that the tumor characterizes itself as a 'nonself' tissue, thus attracting the attention of the organism's immune system and thereby inviting its own destruction. The fact that genes and pseudogenes are susceptible to this type of activation is, in some sense of the word, a function, and one that resembles the function of a burglar alarm. Whereas it is to the tumor cell's advantage to remain invisible to the organism's immune system by behaving much like a healthy tissue cell. The organism can at least potentially benefit by its immune system detecting an alarm signal that has been triggered by a 'misbehaving' cell. In fact, most tumors are believed to take advantage of this type of invisibility.[27] However, alternative open reading frame usage is one mechanism that can force tumors to shed this cloak of invisibility. Of course, from a teleological point of view, we can reasonably deduce the fact that God did not intend cancer to exist as part of His original creation, and this allows us to envision a once-perfect pre-Fall immune surveillance system that never failed to locate and destroy potentially deviant cells.

Why, then, do tumors commonly avoid destruction by the immune system? Apart from often possessing immunological invisibility, tumors avoid destruction by taking advantage of an immune system weakened by carcinogens, by refraining from activating the co-stimulatory molecules necessary for effective CTL performance, by growing so rapidly that they become too large to handle, or by actively suppressing the immune system.[28]

A subset of T cells, $CD25^+$ regulatory T cells, normally modulate the actions of conventional T cells in order to prevent autoimmune disease. There is evidence suggesting that $CD25^+$ regulatory T cells may become overstimulated owing to the immunosuppressive nature of the tumor environment, thereby inhibiting an otherwise-successful T cell response against the tumor. In fact, a tumor antigen's immunosuppressive cytokines may favour the stimulation

of CD25+ cells.[29] However, the nature of tumorogenic immunosuppressive cytokines is not well understood.[30]

The fact that T cell responses against tumor antigens have experienced limited success does not negate their significance in this regard:

> 'Numerous mouse tumor models have been developed to examine the role of T cells in the eradication of tumors. The overwhelming conclusion from these models is that both CD8+ and CD4+ T cells play a role in the effective eradication of tumors.'[31]

As for humans, the action of CTLs has been shown to be largely responsible for the regression of transplanted tumors.[32] Similar considerations apply to CTLs in the context of prospective anticancer vaccines in humans:

> 'Although in vivo expansion of antigen-specific T cells has been observed in a number of cases following vaccination, there is a poor correlation between T cell responses and measurable clinical effects. Conversely, when remissions are observed they often occur in the absence of measurable T cell activity. Nonetheless, pre-and prospective analysis of patients with *early stage cancers* have revealed evidence of a role for T cells in the control of tumor growth' [emphasis added].[33]

Tumor antigens from alternative open reading frames: recent developments

The apparent lack of follow-up studies relative to *NA88-A* has not been true of other antigenic peptides that originate from unconventional gene behaviour. One area of success, in terms of tumor antigens actually triggering the eventual destruction of the tumor, has been in the case of tumors caused by viruses. Consider MAIDS, the murine form of the AIDS virus. The *in vivo* depletion of CD8+ CTLs has been shown to convert MAIDS-resistant mice to susceptible ones, and the antigenic peptide (having an amino acid sequence of SYNTGRFPPL produced by the alternative open reading frame (ORF2) appears to be directly responsible for the stimulation of these tumor specific CD8+ cells:

> 'Because of the uniform activity [lysis of cells] against targets capable of expressing the ORF2-defined CTL epitope, including synthetic peptide pulsed P815B target cells, but not target cells infected with SIN:dG9208-225, which does not encode the ORF2 epitope, these results were consistent with SYNTGRFPPL as the major specificity of the F_1 anti-gag CTL.'[34]

We still do not fully understand the reasons for the inadequate CTL response to many tumors. We do realize, however, the need for the *in vivo* production of those co-stimulatory molecules and helper cells necessary for the CTLs to execute a successful eradication of tumor cells. Recent research on two antigenic peptides, NY-ESO-ORF2 and CAMEL, each the product of an alternative open reading frame, helps us understand how a successful immune response against a tumor cell can be mounted. Particular significance is attached to the *in vivo* production of Th2 helper cells (and associated interleukins) and their relationship to these two antigenic peptides:

> 'Both NY-ESO-ORF2 and CAMEL have been demonstrated to be immunogenic in vivo, since CTL directed against these alternatively translated proteins have been isolated from melanoma patients [Ref.] … By screening a panel of melanoma patients we provide strong evidence for the occurrence of Th2 responses against CAMEL, a tumor Ag [antigen] translated in an alternative ORF. Furthermore, this is the *first report* that describes the isolation and characterization of CD4+ Th2 clones specific for an identified tumor Ag … We propose that presentation of the newly identified MHC class II-binding CAMEL epitope in combination with the previously described MHC class I-binding peptides by well functioning DC [dendritic cells] might be an effective antitumor vaccine'[35] [emphasis added].

There is also some evidence suggesting that a certain tumor antigen, BING-4, synthesized from an alternative open reading frame of a gene, is associated with a successful immune response against the tumor:

> 'The present study was undertaken to obtain clues to explain the factors important in patients undergoing regression of metastatic cancer following immunotherapy. The reactivity of patient TF to the NY-ESO-1 Ag as well as to the new BING-4 tumor Ag in the absence of any deliberate immunization to these Ags suggest that these reactivities may have played a role in the tumor destruction.[36]

What other surprises are in store from the products of alternative open reading frames? And wouldn't it be ironic if pseudogenes, supposedly nothing more than defective copies of genes, turned out to play a role in protecting the organism from tumor cells?

An immunobiological function for intergenic junk DNA?

Our discussion is now expanded to encompass the bulk of the DNA found in our genome. For decades evolutionists had been making assertions about the uselessness of as much as 95% of human DNA. Why is there so much of it? Forsdyke *et al.*[37] have recently suggested an overall function based largely on three observations:

1. The surprising existence of an order of magnitude more transcription of overall DNA than can be accounted for by its contained genes,[38]
2. The tendency of long stretches of intergenic DNA to be either enriched in purines or pyrimidines (purine-loaded and negatively purine-loaded, respectively).
3. The ability of partially complementary strands of RNA to form a complex of dsRNA (double-stranded RNA),

at times serving as a regulatory mechanism. (Recall the existence of a functional snail pseudogene[2] the RNA transcript of which is partly complementary to that of its paralogous gene, thus regulating the gene's expression by effectively removing its RNA from the cytosol through the formation of a strand of dsRNA).

It is considered that the copious transcripts from noncoding DNA are simultaneously diverse enough to ensnare 'nonself' viral transcripts (via dsRNA), yet organized enough (purine loading) to avoid self-on-self dsRNA complexing. The proposed function of bulk junk DNA is summed up as follows:

> 'Formation of dsRNA has long been recognized as an early cellular response to viral entry. Protein synthesis can be inhibited nonspecifically by very low concentrations of dsRNA . . . . If a virus introduced its own RNA into a cell, would there be sufficient variability among host RNA species for a host 'immune receptor' RNA to form a segment of dsRNA with the 'non-self' RNA of the virus? Calculations made elsewhere [Ref.] show this to be feasible, *especially if the entire genome were available for transcription*' [emphasis added].[39]

This suggests that, not only is virtually all noncoding DNA functional to some degree, but the great abundance of this intergenic DNA is *itself* an integral part of its immunological function. In terms of specifics, the large amount of variance collectively present in all of our noncoding DNA makes it likely that virtually any 'nonself' viral transcript will encounter a transcript (from the host's DNA) capable of forming a dsRNA complex with it, thereby slowing down the process of viral protein synthesis. The extensive variance contained in bulk DNA, in effect, adds up to a difficult 'moving immunological target' against infective viruses:

> 'High polymorphism of nongenic DNA would make it difficult for viruses to anticipate the 'immune receptor' repertoire of future hosts.'[40]

No wonder humans, and many other organisms, have so much noncoding DNA!

The aforementioned 'purine loaded' and 'negative purine loaded' patterns in much of bulk DNA apparently facilitate 'self'-tolerance. They make it unlikely that a transcript from one part of the host's DNA would fortuitously be able to form a dsRNA complex with the transcript from another part of the host's DNA:

> 'Among the RNA species of a cell there might be two whose members, by chance, happened to have enough base complementarity for formation of a mutual duplex of a length sufficient to trigger alarms. Thus there would have been an evolutionary selection pressure favoring mutations in host RNAs that decrease the possibility of their interaction with other "self" RNAs in the same cell. In many cases, mutations to a purine would assist this because purines do not pair with purines.'[39]

It is easy to see that the purine abundance patterns in noncoding DNA are a type of higher-level structure that appears to serve a function. Of course, in the light of special creation, the purine abundance patterns within intergenic DNA would be the product of deliberate design instead of naturally selected accidental mutations.

Conclusions

For the longest time, evolutionists had supposed that only genes' DNA is functional. The apparent lack of sequence conservation (from organism to organism) that is generally true of pseudogenic DNA and intergenic DNA was taken as proof that it is merely junk that has been steadily accumulating random mutations. The evidence presented in this report provides a further basis for rejecting this common belief. Noncoding DNA can have one or more immunological functions despite having a low degree of sequence specificity. In the case of pseudogenes, a very short open reading frame can produce potentially useful peptides. This confirms and extends earlier observations.[2,3] More research is needed to clarify the functions of tumor antigens in fighting cancer as well as the production of such antigens from the unconventional reading frames of both genes and pseudogenes.

The functional, and potentially functional, pseudogene products can be put into a broader context. Now more than ever, there is a need to heed the warning about assuming that some seemingly useless gene product is indeed useless:

> 'At the same time, it is also not safe to dismiss a given form as 'functionless' simply because it has no obvious function. For example, even an alternative splice form that causes early translational termination (and an inactive protein product) can act as an important form of regulation of biological activity [Ref.]. Only detailed functional studies can resolve these questions.'[41]

Need any more be said?

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