Mutagenic Selectivity for the RNA-forming Genes in Relation to the Carcinogenicity of Alkylating Agents and Polycyclic Aromatics

O. G. Fahmy and Myrtle J. Fahmy

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, SW3 6JB, England

SUMMARY

Selective mutagenicity was studied in Drosophila for 2 groups of carcinogenic chemical series with divergent intrinsic reactivities towards cellular macromolecules: (a) the reactive alkylating and nitroso compounds; and (b) the nonreactive polycyclic hydrocarbons, aromatic amines, and azo dyes. Genetic activity with each compound was examined with respect to the specific effects on the RNA-forming genes (yielding bobbed and Minute mutations) and the nonspecific overall genetic damage as indicated by the X chromosome recessive lethals and visibles. General mutagenic activity on the X chromosome was invariably high with compounds that can effect biological alkylation, but it was extremely low, or nonexistent, with the nonreactive series.

The levels of mutagenic selectivity for the rRNA loci with the different compounds were measured as the ratios of their yields of bobbed mutants relative to the overall X mutations induced in the same sample of treated gametes. These ratios varied significantly for compounds within each of the reactive and nonreactive chemical groups, but the differences were much more marked between them, being dramatically higher for the polycyclic amines. Carcinogens among all chemical series proved highly active on the RNA-forming loci, particularly the rRNA genes. This was largely or exclusively due to target selectivity for the strongly carcinogenic hydrocarbons and aromatic amines but was partly a function of the general high mutagenic activity for the oncologically active representatives among the alkylating and nitroso compounds.

INTRODUCTION

The role of mutations in carcinogenesis has been a subject for extensive investigation for well over 20 years, and the main developments have sporadically been reviewed and evaluated (1, 4, 10). It became increasingly evident that there was no marked correlation between the oncological efficiencies of chemical carcinogens and their overall mutagenic activities as measured in a diversity of genetic test systems ranging from viruses to mammals. The problem appeared in an entirely new light, however, as a result of detailed genetic studies in Drosophila (9—14) aimed at the analysis of the mutational spectra of chemical series known to include strong carcinogens, particularly the polycyclic hydrocarbons, aromatic amines, and azo dyes. The oncologically active compounds within such series exerted a characteristic type of selective mutagenicity which was virtually restricted to heterochromatic loci involved in the transcription of RNA: the rRNA genes (27), which mutate to bobbed (bb), and the tRNA sites (26, 33, 42), the deletion of which results in Minutes (M's).

The induction of mutations at the RNA-forming genes by some classical carcinogens raised the question as to whether the phenomenon involved some degree of target selectivity by the oncologically active compounds themselves or was merely a manifestation of the general vulnerability of the responsive loci to all mutagens. Evidence for the selectivity interpretation was obtained with the polycyclic hydrocarbons (13), where it was found that the strongly carcinogenic derivatives among the methylated benz (a) anthracenes were appreciably active on the rRNA sites (yielding bb's), whereas their noncarcinogenic analogs were ineffective on these loci. More significant were the relative mutability results for the RNA sites as compared to other euchromatic genes in response to highly mutagenic levels of a diversity of alkylating and nitroso compounds (14). Mutagenically equivalent doses of these compounds with respect to the overall X mutations (recessive lethals and visibles) yielded very different mutation frequencies at the RNA loci (both with respect to the bb's and M's), which argued against the general mutagenic sensitivity of these genes.

It appeared, therefore, that there were sufficient indications for the preferential mutagenicity of some carcinogens on the RNA genes to warrant further investigation of the problem. This was approached in the present study by the detailed analysis of the levels of mutagenic activity and degrees of selectivity at the RNA genes after biological alkylation as compared to that with the intrinsically nonreactive carcinogens among the hydrocarbons and aromatic amines. The tested alkylation agents included some nitroso compounds and were of variable chemical reactivities and carcinogenic efficiencies, but all were effective mutagens for which reactions with cellular macromolecules, especially DNA, had been extensively investigated (20—25). It was thus hoped that the genetic results might help in the elucidation of the molecular mechanism of carcinogenesis, at least as to how far the extent and sites of DNA alkylation were of relevance to the initiation of cancer.
MATERIALS AND METHODS

The analysis of mutagenic response at different sites within the genome was undertaken on the germ cells of Drosophila melanogaster by the genetic techniques devised in our laboratory and used in previous comparable studies (9, 11-14). It is based on the determination of the mutation frequencies at specific eu- and heterochromatic loci relative to that for the whole X chromosome and measured as recessive lethals and visibles.

The Marker Genes. The euchromatic loci used in the analysis of specific mutability were all sex-linked recessive visible mutations of very clear phenotypic expression and were selected to demarcate the whole euchromatin of the X chromosome: white (w, 1.5), forked (f, 56.7) and maroon-like (mal, 64.8). The tested heterochromatic loci were those previously shown (9-14) to be preferentially mutated by carcinogens: the autosomal dominant Minutes (M's), which are represented by several sites within the genome, and the sex-linked recessive bobbed (bb, 66.0), localized in the nucleolar organizer (N.O.) region of the X chromosome, proximal to the centromere.

The molecular basis of mutations at the tested heterochromatic loci (M's and bb's) has been revealed by nucleic acid hybridization studies in Drosophila (26, 27). Evidence was adduced that the genome of this organism contains some 55 to 60 wild-type M alleles, which were probably the sites of transcription of tRNA (26). This was later confirmed by in situ tRNA/DNA hybridization studies on the giant salivary gland chromosomes, with the use of tritiated tRNA and autoradiography (33, 42). The sites of hybridization along the chromosomes, as indicated by radioactive labeling corresponded to the M loci, as had been previously determined by classical cytogenetic techniques. More direct and decisive results were obtained by the nucleic acid hybridization technique as to the function of the bb locus (27). These studies with several bb alleles revealed that such mutations arose as various length DNA deletions within the N.O. heterochromatogic segment of the X chromosome, which resulted in a substantial reduction in the cellular output of rRNA, amounting to about 40% of that of the wild type for mutants with extreme phenotypic expression. The marker bb used in this study was one of the alleles actually analyzed biochemically and was found to reduce the cellular rRNA in the homozygous state. It thus follows that mutant alleles recovered against it experimentally must have involved an almost equal reduction in rRNA content, so as to result in a degree of phenotypic expression similar to that of the homozygote.

Mutation Assays. The overall X recessive mutations were determined by the Muller-5 technique according to a special method of F2 scoring (8), which enabled complete assay of the lethals, including most gonadal mosaics.

Mutagenic treatment was given to Y-bb males of wild-type phenotype and were accordingly free from any background bb mutants on their X chromosomes. The compounds under test were administered in solution by microinjection into the hemocoelic cavity around the testes at an average volume of 0.25 ± 0.05 μl/male. The treated males were mated to females heterozygous for a triple marker X chromosome (f, malbb, bb) and a Muller-5 (M-5) homolog carrying w0;sc81B (In S) w0 sc8. Any induced specific effects would be detectable in the F1 female progeny: possible mutants to M and w in the heterozygous M-5 class and those to M, f, mal, and bb in the complementary triple marker heterozygotes (potentially wild type). A sample of the F1 nonmutant heterozygous M-5 females was then bred on for the detection of the overall X recessive lethals and visibles. The mutagenic activities of the various compounds were assayed for all stages of spermatogenesis by the same progeny fractionation (or "brooding") technique. The treated males were repeatedly mated to the appropriate virgin females at a standard mating pressure (3 females per male) and regular time intervals (2- to 5-day broods) for a total of not less than 15 days after treatment to ensure the sampling of the spermatogonia.

Special precautions were taken in the initial phenotypic scoring of all "visible" mutations, particularly the M's and bb's, to minimize the subjective element in their detection. A stable bb marker of clear phenotypic expression was used in the specific mutability tests at this locus to ensure maximal expressibility among the newly arising mutations (11, 14). The initial detection of the bristle mutations (whether bb or M) was undertaken by highly experienced workers, thoroughly familiar with the range of variability in their phenotypic expression. Instances involving slight reduction in bristle size were ignored unless they could be confirmed unequivocally by subsequent genetic testing. Mutagenicity assays were undertaken concomitantly for the control and treated series, and all the constituent test cultures were coded by a nonobserver. The coded cultures were distributed at random between the scorers, and their decoding was left until the end of experimentation. The results obtained by different workers were invariably statistically homogeneous and were accordingly pooled.

The phenotypes of the bb's and M's are virtually identical, thus necessitating the provisional scoring of the probable newly arising instances collectively as phenotypic (bb + M). However, the transmissible instances among these aberrations were subsequently classified into the definitive mutational classes by appropriate genetic testing (12, 14). Suspected mutations among F1 females heterozygous for the triple marker (+f malbb, bb) were backcrossed to the same bb allele to distinguish between the bb and M mutants; the former would be expressed homozygously in the F2 females, the latter segregating dominantly throughout the progeny. Bristle effects in females without a bb marker (+/M-5) were likely to be M mutations and were crossed to wild-type males for confirmation. Apparent mutations at the euchromatic sites were bred on to determine their transmissibility and were confirmed by allelism.

RESULTS

Interlocus Mutability after Biological Alkylation. The study of mutagenic sensitivity at the bb and M loci relative to other genic sites was undertaken with some of the best known mutagens among the alkylating and nitroso compounds. The alkylating agents were represented by MG2 and its

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2The abbreviations used are: MG, bis(2-chloroethyl)sulfide (mustard gas); hMG, ethyl-2-chloroethyl-sulfide (half-mustard gas); MNNG, N-methyl-N-nitro-N-nitrosoguanidine; ENNG, N-ethyl-N-nitro-N-nitro-
monofunctional analog hMG; the nitroso compounds were represented by MNNG and its ethyl analog ENNG. All compounds were administered at toxicologically equivalent doses (about the LD₃₀ level), and care was taken to avoid their deterioration outside the target tissue (the testes). The mustards were expected to be speedily hydrolyzed in water and were accordingly administered in a DMF oily mixture (2% v/v DMF in dried Arachis oil). This precaution was unnecessary for the nitrosamidines (MNNG and ENNG), because of their much slower hydrolysis rates in neutral aqueous solutions, and they were accordingly dissolved and injected in 0.4% w/v aqueous NaCl.

The sulfur mustards proved to be mutagenically active on all the investigated loci at the dose levels used (Table 1). The difunctional agent (MG) produced a high level of general mutability (111/1000 X chromosomes) at low injected doses (0.3 to 0.4 mM). At this mutagenic level, it was also appreciably effective on the bb and M loci, as indicated both by the collective yield of the phenotypically scorable apparent mutants and the frequencies of the genetically proven instances for each mutational class. The same treatment also mutated the 3 tested euchromatic loci at a mean transmissible frequency per locus which was about 1 order of magnitude lower than for either the bb’s or M’s. The absolute mutagenic efficiency of the MG treatment on the euchromatic loci was indicated by the specific mutation frequencies at 2 of the investigated genes: f = 4.0 ± 1.8 and w = 3.4 ± 1.7 per 10⁶ treated gametes. These frequencies are within the range previously obtained for the same genes with mutagenically effective levels of X-irradiation (32, 38-40). This shows that the mutagenicity of MG, as with radiation, was essentially equivalent doses of X-irradiation (32, 38-40). This shows that the mutagenicity of MG, as with radiation, was essentially random, at least with respect to activity on the euchromatic loci. The disproportionately higher mutability with this agent as regards the bb’s and M’s, therefore, could either be a manifestation of the higher sensitivity of these loci or an outcome of their larger mutagenic target, due to their representation by several mutable sites. There is no evidence for the sensitivity interpretation, but it is well known that the M’s are represented by several loci (estimated at 55 to 60 (26)) which are distributed throughout the chromosome complement. Likewise, the complexity and high redundancy of the bb region has now been demonstrated by both biochemical (27) and genetic (28) techniques. There is little doubt, therefore, that the higher mutation frequencies for each of these loci relative to the average per euchromatic locus (about 10-fold for MG) could easily be accounted for on the basis of their multiple representation within the genome.

The monofunctional sulfur mustard (hMG) proved to be a much weaker mutagen than its difunctional analog with respect to all the examined genetic functions (compare MG and hMG, Table 1). The overall frequency of X recessive mutations with the LD₃₀ dose of hMG (5 mM) was about one-half that occurring with less than one-tenth the injected molarity of MG, indicating some 20-fold higher mutagenicity for the difunctional compound. The activity of hMG on the bb and M loci was also considerably lower than that for MG and...
disproportionately so in comparison to the X mutations, being less than one-fourth for the former as compared to about one-half for the latter. Of the 3 tested euchromatic loci, only f responded to hMG, but again at a lower frequency than that expected for a mutagenically equivalent dose of MG.

The genetic consequences of biological alkylation through reactive species generated in vivo were examined for the alkyl-nitrosoguanidines. Two compounds of this series (MNN and ENNG) proved active on all the investigated loci, but considerable differences in interlocus mutability were noticeable between the methyl and ethyl analogs (Table 2). At roughly comparable mutagenic levels with respect to the X mutations, MNNG gave a considerably higher yield of apparent bristle mutations \( (bb + M) \) than ENNG, but both were less effective in this respect than mutagenically equivalent doses of MG. Essentially the same trends were conveyed by the frequencies of the transmissible \( bb \)'s but not the \( M \)'s, where the yield with MNNG was not significantly above that for ENNG. However, this deviation with respect to the \( M \)'s might be an artifact of mutant recovery with MNNG, since the transmissibility of these mutations for this compound showed considerable variation at different dose levels. Both MNNG and ENNG were decisively active on the tested euchromatic loci, although most of the induced mutants failed to be transmitted, particularly with MNNG. Nevertheless, both the initial and transmitted mutation frequencies at these loci clearly indicated that their response, unlike that of the \( bb \)'s, was significantly higher for ENNG than for MNNG.

Response of the RNA-forming Genes to Different Carcinogenic Series. The mutational spectra with the genetic system used were determined for 4 of the best known carcinogens among the polycyclic aromatics: 2 hydrocarbons, 3-MCH and DMBA; and 2 aromatic amines, AAF and DAB. The different compounds were administered in the same vehicle as the sulfur mustards (DMF/oil), and their genetic testing was undertaken at roughly comparable toxicological levels as measured by the rate of mortality among the males given injections. This was chosen to be around the LD30 dose so as to correspond to that which occurred in the experiments with the alkylating and nitroso compounds.

The mutagenic properties of the polycyclic hydrocarbons are typified by the results with 3-MCH and DMBA (Table 3). The activities of both compounds with respect to the overall bristle effects \( (bb + M) \) and the transmissible \( M \)'s were of the same order as those occurring with the investigated high levels of biological alkylation, but they were considerably higher as regards the transmissible \( bb \)'s (compare Tables 1, 2, and 3). At the same time, these compounds were ineffective on the 3 tested euchromatic loci and produced roughly the same X mutations as among the controls. Essentially the same mutagenicity occurred with the aromatic amines and azo dyes (AAF and DAB, Table 3). They also gave an appreciable yield of phenotypic bristle effects and transmissible \( bb \)'s, but hardly any specific euchromatic mutants or overall X recessives.

The level of mutagenic selectivity for the \( bb \) loci with the polycyclic aromatics relative to that for the alkylating agents could be gathered from the analysis in Table 4. Relative response at the \( bb \) locus with each compound was measured as
the ratio of the transmissible induced number of these mutants relative to the corresponding X recessive lethals and visibles expected in the observed sample of treated gametes. The \( bb \) mutability ratios for the investigated compounds fell clearly into 2 well-defined orders of values. The polycyclic aromatics gave ratios approaching unity or well above this level, whereas those for the alkylating compounds were at least 2 orders of magnitude lower. Less dramatic, although statistically significant, differences were also noticeable in these ratios between agents within each chemical series, indicating some level of \( bb \) selectivity even among closely related chemical compounds. A consideration of the range of variability in the \( bb \) mutability ratios for all the tested compounds (Table 4 Column 7), revealed that the highest value (for 3-MCH) was some 3 orders of magnitude above the lowest (for ENNG).

Significantly, the hydrocarbon with the highest \( bb \) ratio was ineffective on the euchromatic loci, both as indicated by its inactivity on the specifically tested genes (3-MCH, Table 3) and by the statistical insignificance of its induced X mutation frequency (normal deviate = 1.33, \( p = 0.18 \); 3-MCH, Table 4). It would appear, therefore, that mutagenic selectivity for the \( bb \) locus manifested by the polycyclic aromatics could reach virtual specificity with some representatives such as 3-MCH.

The results in Table 4 are of considerable bearing on the problem of the correlation between mutagenesis and carcinogenesis. As in previous studies (chiefly Ref. 10), there were clear indications that general mutagenicity measured as total X mutations could not possibly be correlated with carcinogenicity. The polycyclic aromatics were virtually inactive in this respect, in spite of the fact that they were among the most potent known carcinogens (19). Conversely, the tested alkylating and nitroso compounds all exerted high levels of general mutagenicity, although strong carcinogenicity had only been ascertained for MG (16, 17, 19) and MNNG (5, 7, 29, 31, 34, 35). On the other hand, the order of specific mutagenicity on the \( bb \) loci for the different compounds among both the polycyclic and alkylating series was in complete accord with their carcinogenic efficiency. Most significant is that the higher carcinogenicity of MNNG as compared to ENNG (31) was clearly reflected in their relative mutagenic activities on the \( bb \) loci and that the carcinogenically inactive hMG was also mutagenically ineffective on these genes.

The genetic basis for the high \( bb \) yield was somewhat different for carcinogens belonging to different chemical series; it was largely or entirely due to target selectivity for the polycyclic aromatics, but was also a function of the general high mutagenicity for the oncologically active representatives among the alkylating agents. In spite of this, however, there was a correlation between carcinogenicity and \( bb \) mutability irrespective of the chemical series to which the carcinogens belonged. It would appear, therefore, that the crucial genetic factor in this correlation is the absolute mutation frequency at the \( bb \) loci, irrespective of the way these mutations were produced. If so, the \( bb \) mutability test could prove of immense practical value in the cancer field. It could be used as a fast screening technique for the identification of potential carcinogens in the human environment or at least in the establishment of priorities for the much slower and more laborious carcinogenicity testing.
The demonstration that the genetic activity of carcinogens is mainly expressed in Drosophila as bristle mutations (bb's and M's) which are initially detected phenotypically inevitably introduces a subjective element in the assay of their mutagenicity. It would seem desirable, therefore, to inquire into how far this element could have influenced the differential mutability results described in the present study. The M and bb mutants are known to be associated with various degrees of genetic damage, ranging from small chromosome deletions cytologically demonstrable in the polytene elements of the salivary gland nuclei for many M's to the elimination of short DNA segments or even intragenic point mutations for various bb's. The grades of phenotypic expression among these mutants, including their viability and fertility, are, in general, correlated with the associated order of genetic damage, which could mean that some bristle mutations due to minor deletions or point changes, perhaps among the bb's, might well be virtually unscoreable. This would result in the underestimation of the mutagenicity of carcinogens, which might be differential between agents, as a function of the size of genetic damage they could induce. In the present study, however, the bb's occurring with the alkylating agents (particularly MG) were generally of clearer expression and with lower viability and fertility than those of the aromatic carcinogens (especially 3-MCH), which would seem to indicate that the mutations with the reactive compounds were associated with a higher order of genetic damage. Any depression in the bb mutability assays due to poor mutant expression would accordingly be more pronounced for the aromatic carcinogens than for the alkylating agents. It is conceivable, therefore, that the difference in mutagenic selectivity for the rRNA loci between the 2 chemical series might be even more dramatic than indicated by the present bb mutability results.

The discovery of the selective mutagenicity of carcinogens on the RNA-forming loci has far reaching implications in the elucidation of the molecular and genetic mechanisms of cancer initiation, particularly for agents with intrinsic reactivity towards nucleic acids. Combined biochemical and oncological studies with such agents led to the unequivocal conclusion that the extent of overall alkylation of nucleic acids could not of itself account for carcinogenesis. Thus, the methylation of rat kidney DNA by N-methyl-N-nitrosourea was less than after methyl methanesulfonate (36), although carcinogenic response in this organ only occurred with the nitrosamide. Likewise, no correlation could be found between the extent of alkylation by N-methyl-N-nitrosourea or ENNG. The exact molecular mechanism responsible for this differential mutagenicity is unknown, but it could somehow be associated with the ability of methylating nitrosocompounds to effect alkylation at a wider spectrum of the potential receptive sites in genomic DNA (21, 23).

The most significant feature of the mutagenic activity of carcinogens, in relation to cancer causation, is that it was directed, preferentially or exclusively, toward the RNA-forming loci. Mutations at these genes would therefore be expected to result in the transcription of RNA's of abnormal structure or function and this has, in fact, been shown to be characteristic of cancer. The clearest results in this connection concerned the tRNA, where there were indications for a shift in the relative proportions of the different isoaccepting varieties (2, 37) as well as for the appearance of new molecular species (15) during carcinogenesis. Differences were also discernible between the tRNA's of established tumors as compared to the corresponding normal tissues, which were most marked for Ehrlich ascites cells in mice (18) and Novikoff hepatoma in the rat (3). The foregoing changes in tRNA's were shown to be in

### Table 4

<table>
<thead>
<tr>
<th>Chemical series and compounds</th>
<th>Induced mutation frequencies per thousand&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative induced mutation yields</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>X mutations (f + v)</td>
<td>Transmitted bb</td>
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<tr>
<td>Polycyclic aromatics</td>
<td></td>
<td></td>
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<tr>
<td>3-MCH</td>
<td>0.8 ± 0.6</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>DMBB</td>
<td>2.9 ± 1.4</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>AAF</td>
<td>1.2 ± 0.5</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>DBA</td>
<td>1.4 ± 0.8</td>
<td>1.1 ± 0.2</td>
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<tr>
<td>Alkylating agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>108.7 ± 7.5</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>MNNG</td>
<td>118.0 ± 7.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>hMG</td>
<td>49.2 ± 4.6</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>ENNG</td>
<td>87.6 ± 5.6</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The induced mutation frequencies for the various loci were obtained from the observed experimental values by the subtraction of the control contributions due to the administration vehicles.

<sup>b</sup> The number of X mutants corresponding to the bb's for each compound was deduced from the observed sample size and the induced X mutation frequency.

DISCUSSION
part at least due to their preferential methylation in tumors, as a function of the much higher methylase activity known to be characteristic of cancerous tissues (3, 6). There is no direct biochemical evidence for changes in rRNA during carcinogenesis, but this field still remains largely unexplored (6). Also the oncologically significant mutations in the rRNA loci might not involve large DNA deletions (akin to the stronger bb’s in Drosophila) and would not, accordingly, be expected to depress cellular RNA content to a level that could be detected by the available biochemical techniques. Point mutations at the subunits of the rRNA structural genes would cause qualitative changes in the resulting ribosomes, while damage to the genetic control elements (regulators and/or operators) could cause alterations in the time of synthesis or level of output of the various RNA constituents.

The best evidence that cancer could involve changes in the balanced construction of the cellular RNA complement is provided by the alterations in the activities of many enzymes during carcinogenesis (41) and the various changes in protein synthesis known to accompany this process. The latitude required for this variability could easily be accommodated within the framework of the mutagenic selectivity of carcinogens for the RNA-forming loci. In Drosophila, the X chromosome rRNA locus (mutating to bb’s) was shown to be highly complex (28) and redundant (27), and the tRNA genes (mutating to M’s) are expected to be represented by 64 sites widely distributed throughout the genome (26, 33). Homologous genes are known to be present in still higher cellular genetic systems, including mammals, where the multiplicity of their genic representation would be at least maintained, if not increased. Mutations at any of these RNA-forming cistrons would result in the production of aberrant RNA’s, thus providing considerable scope for variability in the RNA complement of the cell. The abnormal RNA systems in the different mutant cells would be expected to exhibit varied efficiencies in the transcription and translation of the genetic code for the various protein-synthesizing genes, which could lead to any of the metabolic disturbances associated with cancer. Comparable molecular alterations in cellular RNA could conceivably arise from direct chemical attack by carcinogens on cultured cells, which would explain the high frequency of malignant transformation known to occur in invitro carcinogenesis.

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