Pseudouridine Excretion and Transfer RNA Primers for Reverse Transcriptase in Tumors of Retroviral Origin

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ABSTRACT

To evaluate the relationship between pseudouridine increase in biological fluids and retroviral cell transformation, we have studied the effect of retrovirus infection and/or transformation on the rate of pseudouridine excretion by chick embryo fibroblasts. The results show that: (a) pseudouridine excretion by chick embryo fibroblasts transformed by Rous sarcoma virus is several times higher than that of normal cells; (b) this increased excretion precedes by many hours the appearance of the morphological signs of transformation and it is always present when neosynthesized infectious viral particles are released into the culture medium; and (c) pseudouridine excretion was also increased in cells infected by a mutant of Rous sarcoma virus (RAV-1) which, lacking the src gene, does not transform the cells but replicates normally.

To investigate if pseudouridine overproduction is related to an altered turnover rate of specific transfer RNA (tRNA) species which functions as primer of retrovirus reverse transcriptase, the concentration of non-acylated proline-accepting tRNA and non-acylated tryptophan-accepting tRNA, primers of reverse transcriptase of murine leukemia virus and of Rous sarcoma virus, respectively, has been measured, the former in normal and transformed AKR thymus and the latter in normal fibroblasts and in fibroblasts infected by Rous sarcoma virus or by its nontransforming mutant. The results show that in both systems a significant increase of the primer tRNA species occurs in the infected or transformed cells.

INTRODUCTION

Increased urinary levels of modified nucleosides have been found in numerous tumor-bearing subjects (1–8). The types of modified nucleosides found in the urine of cancer patients and the observation that tRNA contains the highest and most varied number of modified nucleosides (up to 20% of the total number of nucleosides are posttranscriptionally modified) suggest that alterations in the structure and/or metabolism of tRNA in transformed cells cause the observed phenomenon. However, tumor cells appear to lack hypermodified tRNA species (9), whereas the same cells show enhanced activity of the enzymes involved in the main tRNA modification reactions, i.e., methylation (10) and pseudouridylation (11).

We have found previously that blood serum pseudouridine, the most abundant modified nucleoside in tRNA, increases in several human neoplasias (more frequently in lymphomas, leukemias, and hepatomas) and precociously decreases when a response to therapy is achieved (12). Similarly in AKR mice bearing lymphomas of viral origin (AKR-MuLV), either spontaneous or transplanted, we found that pseudouridine serum levels are significantly higher than in control mice and are normal in mice bearing chemically induced lymphoid tumors (13). Furthermore in the AKR mice the increase of serum pseudouridine appeared to be an early event, evident in the preneoplastic period (13) when the transforming virus is produced (14) and before histopathological signs of the disease are apparent.

All these findings suggested that the overproduction of pseudouridine, rather than being a general aspect of the neoplastic phenotype, could be related to the alteration of the metabolism of specific tRNA molecules involved in retroviral replication.

To investigate this point we used the AKR system, the etiological agent (AKR-MuLV) of which utilizes cellular tRNA as primer for its reverse transcriptase (15), and the CEF transformed by RSV, which utilizes cellular tRNA as primer for its reverse transcriptase (16).

We addressed two points. (a) Is the increased production of pseudouridine in lymphomatous AKR mice a consequence of viral infection and replication or a response of normal cells to the appearance of neoplastic foci? (b) Is the cellular content of the specific tRNA species that acts as primer of reverse transcriptase altered in the cell transformed by a retrovirus?

The first point was studied by evaluating the excretion of pseudouridine and other nucleosides by CEF infected by RSV or its defective mutant RAV-1 which, lacking the src gene, fails to transform the cells but replicates normally (17). The second point was evaluated by measuring the amino acid acceptance of several tRNA species purified from both normal and lymphomatous AKR thymuses or from normal and RSV- or RAV-1-infected CEF.

MATERIALS AND METHODS

Animals. AKR mice were from colonies established from Jackson Laboratory mice by Drs. C. Gurgo and S. Bridges. Thymuses were excised after anesthesia by thoracotomy, cleaned of adherent tissues, and quickly frozen in liquid nitrogen.

Cells. Primary cultures of CEF were prepared from body walls of 11-day-old White Leghorn chick eggs (Lohman Tierzucht GmbH; Cuxhaven, Federal Republic of Germany) and cultured in a 10-ml/100-mm plastic dish (Falcon, Cockeysville, MD) of E199 medium (Difco Laboratories, Detroit, MI) containing 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum at 37°C in 5% CO2. This incubation procedure was...
used in all experiments. Secondary cultures were seeded at 5 x 10^4 cells/60-mm plastic dish in 3 ml of E199 medium containing 2% tryptose phosphate broth, 1% calf serum and 1% chicken serum and exposed to 0.5 ml of RSV, Schmidt Ruppin strain, subgroup A, or to RAV-1 for 1 h. Control cultures underwent the same procedure, but with 0.5 ml of medium.

After 1 h of incubation, the medium was replaced by 3 ml of fresh medium and incubated as described. In two different sets of dishes (each in quadruplicate), the media were collected every 24 h (one set at 12, 36, 60, and 84 h and the other at 24, 48, 72, and 96 h) and stored at -20°C; 3 ml of fresh medium replaced the original medium. Control plates were treated in the same way for cell counting (in duplicate).

**Virus Production Assay.** To determine RSV and RAV-1 production by infected cells, 0.5 ml of each medium collection (from 36 to 96 h) was used to infect secondary cultures. The appearance of the transformed phenotype indicated the presence in the sample of RSV, whereas the impossibility of obtaining transformation when these secondary cultures were reinfected with RSV indicated the presence of RAV-1 (challenge assay).

**Nucleoside Purification and Analysis.** The purification, identification, and quantitation of major and modified nucleosides excreted by cells in the culture medium were performed according to the methods of Colonna et al. (18), with modifications. Briefly, culture medium samples were centrifuged to remove cells and debris; 1-ml aliquots of the supernatant were deproteinized with 1 ml ice-cold acetonitrile, placed on ice for 15 min, and centrifuged at 3000 x g for 30 min. The supernatant was treated with bacterial alkaline phosphatase (0.75 unit/ml) in 0.1 M Tris-HCl, pH 8.5, for 3 h to convert nucleotides to nucleosides. This procedure eliminates small quantities of GMP that coelute with pseudouridine. The ribonucleosides were purified on a phenylboronate column (Affi-Gel 601; Bio-Rad Laboratories, Richmond, CA) by eluting with 0.1 M formic acid in H2O, and 250-µl aliquots were injected on a C18-Bondapak column (Waters Associates, Milford, MA) using previously described high performance liquid chromatography equipment (18). Nucleosides were eluted at a flow rate of 1 ml/min with 0.01 M NH4H2PO4, pH 5.1, containing 60 µl of methanol/liter and quantitated with a Hewlett-Packard 3390 integrator using deoxyguanosine as internal standard.

**tRNA Aminoacylation Assay.** tRNA-aminocaylsynthetase was purified from wheat germ according to the method of Corbo et al. (19). Decacylated tRNA from both AKR thymuses and CEF (confluent secondary cultures) was prepared as follows. Cells or tissues were homogenized in 2 volumes of 0.14 M NaCl:0.05 M sodium acetate, pH 5.1:1% sodium dodecyl sulfate, and centrifuged at 10,000 x g for 1 h; the supernatant was centrifuged at 105,000 x g for 90 min. Clear supernatant was treated in the same way for cell counting (in duplicate).

**Aminoacylation reaction was performed as described (19) using [3H]amino acids (Radiochemical Centre, Amersham, Buckinghamshire, England) at a specific activity of about 2.5 Ci/mmol, in a final volume of 40 µl, using 0.2 A260 units of tRNA, 40 µM [3H]amino acid, and 0.15 µg of enzyme in 30 µl.

**RPC-5 Chromatography.** Two A260 units of tRNA were aminoacylated as described and applied onto a 0.7- x 80-cm column of RPC-5 and eluted with a linear gradient from 0.3 to 1 M NaCl in 0.01 M sodium acetate:0.01 M MgCl2:0.001 M EDTA:0.002 M β-mercaptoethanol, pH 4.5. Fractions of 1 ml were collected and the radioactivity of the whole fraction was counted in 3 ml of Insta-Gel in a Packard Tri-Carb liquid scintillation system (20).

**RESULTS**

**Pseudouridine Excretion by Chick Embryo Fibroblasts.** The chromatographic profile of the nucleosides purified from the culture medium of CEF is shown in Chart 1. Pseudouridine, cytidine, uridine, inosine, guanosine, and adenosine were identified by comparing retention times and absorbance ratios (A254/A280) of the unknown peaks with those of pure compounds chromatographed under the same conditions. The abundance of inosine is probably due to adenosine deaminase contained in the calf serum present in the culture medium.

The concentrations in culture medium of the major nucleosides at various times after RSV or RAV-1 infection were very similar to those of normal CEF (data not shown) and consistent with the data reported by Uziel and Selkirk (21). A dramatic quantitative difference was detectable for pseudouridine excretion. Chart 2 depicts the time course of pseudouridine excretion in normal and infected CEF. A significant (P < 0.02) difference between normal and infected cells was already evident 60 h after RSV infection. The production of viral particles was evident at 60 h after the infection, and at 72 h the difference between pseudouridine excretion of normal and infected cells was maximal, even though no morphological sign of the transformed phenotype was detectable as yet; this latter began to appear only 84 h after viral infection.

RAV-1-infected cells show amounts of pseudouridine excretion very similar to those observed in RSV-infected cells. The time of appearance of neosynthesized viral particles in the medium was identical to that of RSV-infected cells; obviously no sign of transformation was observed in these cells.

**tRNA Isoaccepting Species in Normal and Transformed Cells.** Because the tRNA primer assembled in the de novo-produced viral particles is of host cell origin (15, 16), we deter-
detected its concentration in two experimental systems for which the tRNA primer for reverse transcriptase is known (tRNA\(^{\text{Pro}}\) for AKR-MuLV and tRNA\(^{\text{Trp}}\) for RSV). As reported in Table 1, proline acceptance by tRNA from lymphomatous AKR thymus is 4-fold higher than that of normal (2-month-old mice) AKR thymus tRNA, whereas tRNA\(^{\text{Trp}}\) and tRNA\(^{\text{Tyr}}\) were unchanged. To exclude that the observed increase could be due to the presence in transformed cells of "new" tRNA\(^{\text{Pro}}\) species with higher affinity for the specific aminoacylsynthetase, reverse phase chromatographic analysis on an RPC-5 column of acylated tRNA was performed (see Chart 3). The close similarity in the retention volumes of tRNA\(^{\text{Pro}}\) peaks between normal and lymphomatous AKR thymus suggests an increase of normally present tRNA species in transformed tissue.

Similarly in CEF a 2-fold increase of the specific tRNA primer, tRNA\(^{\text{Trp}}\), was observed in RSV-transformed cells (see Table 1). Such an increase was also present in RAV-1-infected CEF. Also in this case the chromatographic elution profiles on RPC-5 excluded the presence of a "new" tRNA\(^{\text{Trp}}\) species because they showed a single peak, with identical chromatographic properties (data not shown).

**DISCUSSION**

Our previous studies suggested that pseudouridine can be considered a marker of viral cell transformation. In fact the increased pseudouridine serum levels in mice with lymphomas of viral origin before the appearance of the neoplastic phenotype and the absence of this increase in a murine chemically induced lymphoma supported the hypothesis that pseudouridine overproduction is not a general phenomenon of neoplasias but a specific consequence of one of the steps of retrovirus-cell interaction (13). This hypothesis is strongly supported by the present report of an overproduction of pseudouridine in RSV-infected CEF many hours before the appearance of the transformed phenotype. Moreover increased pseudouridine excretion was evident also in RAV-1-infected CEF, in which the mutant virus does not transform the cells but replicates normally, therefore causing virus particle production. The two experimental systems, i.e., AKR mouse strain and CEF transformed by RSV, have similar properties. In both cases an oncogenic retrovirus is the etiological agent of cell transformation, and both viruses use a cellular tRNA as primer for reverse transcriptase. The tRNA primers for MuLV and RSV have been identified as tRNA\(^{\text{Pro}}\) and tRNA\(^{\text{Trp}}\), respectively, and completely sequenced (15, 16). Incidentally they both contain in loop IV an extra pseudouridine residue instead of the normally present ribothymidine (15, 16); this means that their pseudouridine content is four and three residues per molecule, respectively.

We found a significant increase of the concentration of the two specific primers, tRNA\(^{\text{Pro}}\) in lymphomatous AKR thymus and tRNA\(^{\text{Trp}}\) in RSV- or RAV-1-infected CEF. The finding that also in

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**Table 1**

<table>
<thead>
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<th></th>
<th>Normal</th>
<th>Lymphomatous</th>
<th>Normal</th>
<th>RSV</th>
<th>RAV-1</th>
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<td>tRNA(^{\text{Pro}})</td>
<td>8.75</td>
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<td>5.10</td>
<td>5.25</td>
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<td>7.80</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) Charged tRNA amount was calculated on the basis of amino acid specific activity.
RETROVIRUSES AND LEVELS OF PSEUDOURIDINE AND tRNA

RAV-1-infected CEF there is an increase of tRNA\textsuperscript{0} indicates that the phenomenon is not linked to the transformed phenotype, because RAV-1 lacks the src gene. The identity of the elution profiles of \textsuperscript{3}H-labeled tRNA\textsuperscript{0} from normal and lymphomatous thymus as well as of \textsuperscript{3}H-labeled tRNA\textsuperscript{0} from normal and RSV-infected cells seems to rule out the possibility that the increased concentration of these tRNA species could be due to the appearance of "new" isoaccepting tRNA, as described in other systems (22, 23). It is worth noting that the proportion between the two isoaccepting species of tRNA\textsuperscript{0} seems to be slightly changed in transformed cells.

During the assembling of neosynthesized viral particles, the tRNA primer is the only component not coded for by the viral genome and it must be supplied by the host cell (24). This could indicate that a more active synthesis of a specific tRNA species occurs in the host cell because a consistent aliquot of the primer molecules is entrapped in neosynthesized viral particles. Specific activation of the transcription of particular tRNA species may also be speculated.

The latter hypothesis is supported by the observation that cultured fibroblasts contain much more tRNA\textsuperscript{0} than do thymocytes (see Table 1). This is probably due to the preponderant translation of proline codon-rich collagen mRNA, which requires an elevated number of adaptor molecules. Furthermore in the RSV-transformed cells a decrease of tRNA\textsuperscript{0} is observed, and it seems to be related to the decrease of the collagen mRNA transcription (25).

We are now attempting to identify and clone the tRNA\textsuperscript{0} and tRNA\textsuperscript{0} genes to study the mechanisms that control their expression in the two experimental systems. As yet no mechanism is known for the regulation of the tRNA gene expression in eukaryotic cells (26).

The data presented here suggest that the increased production of pseudouridine could reflect the increased turnover of the specific tRNA the synthesis of which is enhanced in the cell as a consequence of retroviral replication. Any increase of modified nucleosides other than pseudouridine (e.g., 1-methyladenosine, 1-methylguanosine, 2-methylguanosine, etc.) cannot be detected because they are present in tRNA\textsuperscript{0} in amounts 3-fold lower than is pseudouridine and are therefore below the minimal detection limit of the high performance liquid chromatography procedure (18) used (25 pmol of nucleoside). Moreover these compounds are also excreted as modified bases because they possess an N-glycosidic bond that is subject to hydrolysis, whereas the C-glycosidic bond present in pseudouridine is highly stable (27) and thus only this nucleoside is a true end product. The same applies to modified nucleosides in the blood serum of cancer patients (12) and of AKR lymphomatous mice (13). However, in our previous studies (4) on human lymphomas we found that increased pseudouridine urinary concentration was accompanied by an increase of other modified nucleosides, such as 1-methyladenosine, 2-methylguanosine, 1-methylguanosine, and 7-methylguanosine. Similar results have also been reported by other authors (2, 5, 8).

The evaluation of pseudouridine as an etiologic marker for neoplasias where retroviruses are implicated is a promising area for further experimental studies.

REFERENCES


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