Studies on Arginyl Transfer Ribonucleic Acid in Herpes Virus Infected Baby Hamster Kidney Cells

By D. BELL*, N. M. WILKIE* AND J. H. SUBAK-SHARPE
Institute of Virology, University of Glasgow, Glasgow, W. 2, Scotland.

(Accepted 11 August 1971)

SUMMARY

Previous work, (Subak-Sharpe & Hay, 1965, Subak-Sharpe, Shepherd & Hay, 1966), depending on the hybridization of [32P]4s RNA from herpes virus infected cells to herpes DNA and analysis of the aminoacyloligonucleotide fragments produced after RNase T1 digestion of [14C]- and [3H]-arginyl tRNA, suggested the presence, in infected cells of a herpes-specified arginyl tRNA. The present study indicates that these results were due to the presence of (a) radioactive RNA other than tRNA in the [32P] 4s RNA, and (b) radioactive contaminants in the [3H]-arginine used to aminoacylate the tRNA. Further purification of the aminoacyl tRNA removes these [3H]-arginine contaminants. Protection of the aminoacyl tRNA ester bond by N-acetylation improved the sensitivity of the aminoacyloligonucleotide analyses and permitted tRNA-specific molecular hybridization. Using these techniques, no herpes-specified arginyl tRNA was detected in cells 7 to 9 hr post infection.

INTRODUCTION

Several investigators have reported the presence in bacteria infected with phage T4 of new species of tRNA which are coded for by the phage genome (Hsu, Foft & Weiss, 1968; Scherberg et al. 1970; Daniel, Sarid & Littauer, 1970). It was shown that aminoacyl tRNA labelled only in the amino acid moiety, would hybridize specifically with phage DNA. In this way it was possible to identify phage T4-specified leucyl, arginyl, isoleucyl and possibly tyrosyl tRNAs.

Earlier work, depending on DEAE chromatography of T1 RNase digests, and hybridization of [3P]-labelled 4s RNA to virus DNA had suggested the presence of virus specified arginyl tRNAs in herpes-infected BHK cells (Subak-Sharpe & Hay, 1965; Subak-Sharpe, et al. 1966). We have re-examined this problem using the improved methods of aminoacyloligonucleotide analysis and molecular hybridization which have since become available.

During the course of this work it was shown that the arginyl and seryl tRNAs from herpes infected and uninfected HEP-2 cells could not be distinguished by reverse phase chromatography (Morris, Wagner & Roizman, 1969). More recently Morris, Wagner & Roizman (1970) reported that generally labelled arginyl and seryl transfer RNAs from herpes infected cells did not hybridize to herpes DNA.

It has also been reported (Raška, Frohwirth & Schlesinger, 1970) that [3H]-arginyl tRNA from adenovirus infected KB cells failed to hybridize to adenovirus DNA.

* Members of the Medical Research Council Virology Unit.
METHODS

Cells and viruses

**BHK cells.** BHK 21 (C13) cells (Macpherson & Stoker, 1962) were grown in monolayer cultures in slowly rotating 80 oz. bottles (House & Wildy, 1965), each of which yielded around 4 x 10^8 cells at confluence.

**Herpes virus infection.** Non-confluent monolayers of BHK cells (2 x 10^8 cells/bottle) were infected with herpes simplex virus (strain a) at a m.o.i. of 10, the virus being assayed at the time of infection. The cell sheet was infected with 20 ml. of virus suspension and the bottle rotated at 37° for 1 hr. 50 ml. of medium was then added and the incubation continued for a further 7 to 9 hr.

**Escherichia coli.** E. coli B were grown with aeration at 37° in synthetic TCG medium (Kozinski & Szybalski, 1959).

**Phage T4 infection.** E. coli were infected at 37° with T4 phage, at a m.o.i. of 5, super-infected at the same multiplicity 5 min. later and incubated with aeration for a further 30 min. For the details of phage purification and assay, see Ritchie & Malcolm (1970).

Preparation of tRNA

**From BHK cells, or herpes infected BHK cells.** The cells were harvested by trypsinization and the trypsin inactivated by the addition of 1/10 vol. of calf serum to the cell suspension. The cells were collected and washed twice, in the cold, with high salt buffer (HSB: 0.15 M-NaCl, 0.01 M-MgCl_2, 0.05 M-Tris + HCl, pH 7.4) by centrifugation. They were resuspended in cold low salt buffer (LSB: 0.05 M-NaCl, 0.01 M-MgCl_2, 0.05 M-Tris + HCl, pH 7.4), about 100 ml. for 10^10 cells, and allowed to stand at 0° for 10 min. In some experiments, bentonite, to give a concentration of 0.05 %, was added at this stage. The cells were ruptured at 0° with a teflon pestle in a glass Potter-type homogenizer and the resulting suspension centrifuged at (2250g) for 10 min. to remove nuclei, cell debris, etc. When bentonite was not used, diethylpyrocarbonate (Baycovin, Bayer, Leverkusen, Germany, 0.1 % (v/v) final concentration) was added to the supernate at this stage. This was extracted twice with an equal volume of LSB-saturated phenol containing 0.1 % (w/v) 8-hydroxy quinoline and the layers separated by centrifugation. RNA was precipitated from the aqueous layer by the addition of 0.1 vol. of 20 % (w/v) potassium acetate, pH 5.0, followed by 2.5 vol. of ethanol at -20°. The precipitate was left for at least 2 hr at -20°, collected by centrifugation at 1200g for 5 min., washed with 70 % ethanol/LSB and dried in vacuo. The RNA was dissolved in LSB, applied to a DEAE-cellulose (Chloride) column and eluted with a NaCl gradient from 0 to 1 M in LSB. The u.v. extinction peak which eluted around 0.6 M-NaCl was pooled and the RNA precipitated by the addition of 2.5 vol. of cold ethanol. The precipitate was washed and dried as before, dissolved in HSB and stored in 0.5 ml. volumes at -70°.

**From E. coli, or phage T4 infected E. coli.** Chilled cells were collected by centrifugation, resuspended in LSB (about 30 ml. for 2 x 10^11 cells) and extracted twice with an equal volume of LSB saturated phenol/0.1 % (w/v) 8-hydroxyquinoline. The tRNA was then isolated as described above.

The tRNA isolated from virus infected cells, in both systems, was contaminated with small amounts of DNA as judged by alkali resistance.

Aminoacyl tRNA synthetase preparation

**From BHK, or herpes infected BHK cells.** Cells were harvested, washed with cold HSB and suspended at 0° in ASB (0.025 M-KCl, 0.0075 M-MgCl_2, 0.01 M β-mercaptoethanol, 0.05 M-
tris HCl pH 7.4; 10 ml. for about 10⁸ cells). The suspension was treated with ultrasonic vibration (in an ice-bath) for 1 min. (two, 30 sec. periods with 30 sec. between), then centrifuged at 12,000g for 10 min. at 4°C. The supernatant was then centrifuged at 105,000g for 2.5 hr at 4°C. The top 7 to 8 ml. of the supernatant fluid were removed and applied to a Sephadex G75 column (0.8 cm × 25 cm.) equilibrated to ASB at 4°C. The column was washed with ASB and 1 ml. fractions were collected.

The fractions were assayed (in a manner analogous to that described below), for their ability to synthesize arginyl tRNA and the active fractions were pooled and stored as 0.5 ml. volumes at −70°C.

From E. coli. About 1.5×10¹¹ cells were suspended in cold ASB, sonicated at 0°C for 2 min. (in 30 sec. periods with 30 sec. between) and treated as described above for BHK cells, except that the amino acid used in the assay was leucine.

**Preparation of [¹⁴C] or [³H]-labelled aminoacyl tRNA**

The following incubation mixture is typical of those used: 10 μmoles ATP; 4 μmoles CTP; 2 mg. tRNA; 5 μmoles each of the 19 amino acids, other than the radioactive amino acid; 12.5 μC [¹⁴C]-arginine (300 μC/μmole), or 250 μC [³H]-arginine (12 μC/μmole) or 125 μC [³H]-leucine (20 μC/μmole); made up to a final volume of 1 ml. of ASB, to which was added 1 ml. of the aminoacyl tRNA synthetase preparation. This mixture, and an appropriate blank without tRNA, were incubated at 37°C for 10 min. Ten μl. samples were taken after 0 and 10 min. for the determination of acid precipitable radioactivity, by the paper disc method of Bollum (1959). Incubation was stopped by the addition of 20 ml. of ice-cold 0.01 M-sodium acetate pH 5.0. The aminoacyl tRNA was then purified by chromatography on DEAE-cellulose (chloride) at 4°C. Elution was by a linear gradient of sodium chloride 0 to 1.5 M in 0.01 M-sodium acetate pH 5.0. The aminoacyl tRNA was then purified by chromatography on DEAE-cellulose (chloride) at 4°C. Elution was by a linear gradient of sodium chloride 0 to 1.5 M in 0.01 M-sodium acetate pH 5.0. The aminoacyl tRNA was precipitated with ethanol, dried and dissolved in 0.01 M-sodium acetate, at a concentration of about 3 mg./ml. When the radioactive elution profile from the DEAE-cellulose column was required, ammonium formate gradients at pH 5.0 were used. The ammonium formate was removed before counting by drying the fractions at 150°C.

**Preparations of the acetyl ester of N-hydroxy-succinimide**

As described by de Groot et al. (1966) and Lapidot, de Groot & Fry-Shafer (1967).

**Preparation of N-acetylaminoacyl tRNA**

As described by Lapidot et al. (1967) and Daniel et al. (1970).

**Digestion of N-acetyl [¹⁴C]-arginyl tRNA with ribonuclease T1**

N-acetyl [¹⁴C]-arginyl tRNA (usually around 30,000 counts/min., 60 to 80 μg), in 1.0 ml. of 0.15 M-sodium chloride, 0.015 M-sodium citrate, 0.002 M-EDTA pH 7.0, was digested for 105 min. at 37°C with 100 units of ribonuclease T1 (Sankayo Co. Ltd., Tokyo, Japan). Amounts up to 400 units gave identical results.

A small amount of N-acetyl [¹⁴C]-arginyl tRNA (about 5000 counts/min.) was always incubated under the same conditions in the absence of ribonuclease T1, in order to monitor the non-enzymic breakdown of the substrate. Samples (50 μl.) were taken at intervals throughout the incubation for the determination of acid precipitable radioactivity as in § 4 above.
The incubation was stopped by chilling in ice and the addition of 15 ml. 0.01 M-formate pH 5.0. This solution was then applied to a DEAE-cellulose (formate) column, equilibrated to 0.01 M-ammonium formate pH 5.0 at 4°C. The column was washed with 40 ml. 0.01 M-ammonium formate pH 5.0 and then subjected to two linear gradients of ammonium formate, 0.01 to 0.7 M (100 ml./100 ml.). Fractions of approximately 1 ml. were collected, dried down in scintillation vials at 150°C and the radioactivity estimated by scintillation counting using a toluene based scintillator. (This method is not suitable for the estimation of tritium as variable amounts of radioactivity can be lost during the drying process.)

**Preparation of herpes simplex DNA**

BHK cells were infected with herpes simplex virus at a m.o.i. of 1/300 and incubated at 32°C for 3 to 4 days. The cells were shaken off into the medium and centrifuged down at a low speed. The supernatant was then spun at 23,000 g for 3 hr at 4°C. The virus pellet was suspended in 0.15 M-sodium chloride, 0.1 M-EDTA pH 7.4, sodium dodecyl sulphate added to 2% and the mixture immediately heated to 60°C for 10 min. After cooling CsCl was added (1.2 g/ml.), and the solution centrifuged at 12,000 g for 10 min. This yielded a protein pellet over a clear solution. The clear solution was removed, its refractive index adjusted to 1.40, and then it was centrifuged at 105,000 g for 3 days. The resultant gradient was fractionated by dripping and the DNA bands located by their u.v. extinction. The herpes DNA band, buoyant density 1.725, was pooled and dialysed against three changes (3 l. each) of 0.015 M-sodium chloride, 0.0015 M sodium citrate in the cold.

**Preparation of phage T4 DNA**

Phage T4 was purified by differential centrifugation and banding in CsCl and the DNA isolated by phenol extraction as described by Thomas & Abelson (1966).

The phage DNA was finally purified by isopycnic centrifugation in CsCl, as described above for herpes simplex DNA.

**Hybridization procedures**

The filter method (Gillespie & Spiegelman, 1965) was used throughout.

**Preparation of filters.** DNA, at a concentration of 50 to 100 µg/ml., was denatured in 0.165 N-NaOH for 15 to 30 min. at 0°C and then neutralized by the addition of 1.5 N-HCl.

Nitrocellulose membrane filters (27 mm., 40 mm., Schleicher & Schull, supplied by Anderman & Co. Ltd., London) were soaked in 4 x SSC, pH 7.0, for at least 10 min., then each filter was washed by the passage of 50 ml. 4 x SSC, pH 7.0. DNA was applied to the filter at 5 µg./ml. in 4 x SSC, pH 7.0, washed in with 100 ml. 4 x SSC, pH 7.0, and then with 20 ml. 4 x SSC, pH 5.0. The filters were allowed to dry overnight and then baked at 80°C for 2 hr immediately prior to their use. Blank filters were similarly treated but no DNA was added.

The retention of DNA by the filters was always in excess of 80% and usually approached 100%.

**Hybridization and screening.** The prepared filters were immersed for 2 hr at 70°C in 2 x SSC, pH 5.0, which contained the N-acetylaminoacyl tRNA to be hybridized. The filters were then removed and stored in fresh 2 x SSC, pH 5.0 before washing. Each filter was washed with 100 ml. 2 x SSC pH 5.0 at room temperature, incubated for 30 min. at 37°C with ribonuclease T1 (100 units/ml.) in 2 x SSC, pH 6.5, and then washed with 150 ml. 2 x SSC, pH 5.0, at 60°C. The filters were dried and the bound radioactivity determined by scintillation counting in toluene based scintillation fluid.
RESULTS

A previous publication from this laboratory (Subak-Sharpe et al. 1966) reported the presence in T1 digests of tRNA from herpes simplex infected BHK cells, of two new, virus specified, arginyl oligonucleotide fragments. The new peaks eluted before the main peak of host cell material. We believe that these peaks were caused by radioactive contaminants of the [3H]-arginine used to aminoacylate the tRNA. These, as yet unidentified, contaminants can be demonstrated when freshly obtained [3H]-arginine is chromatographed on DEAE cellulose (Fig. 1 a). In this experiment, 2% of the radioactivity was absorbed and could be eluted as two, or more, distinct peaks, at salt concentrations slightly lower than that required

![Graph](image-url)

Fig. 1. (a) Chromatography of [3H]-arginine on DEAE cellulose (formate). 30 μC [3H]-arginine was dissolved in 10 ml. of cold 0.01 M ammonium formate, pH 5.0, and applied to DEAE cellulose (formate) column equilibrated to the same buffer at 4°C. The column was washed with 70 ml. 0.01 M formate, pH 5.0, and then a linear gradient of 0.01 M to 0.5 M (50 ml./50 ml.) applied. The fraction volume was 4 ml. 7.11 × 10^6 counts/min. were recovered in the wash, and 0.15 × 10^6 counts/min. in the peaks shown in the elution profile. (b) Precipitability of the contaminants of [3H]-arginine. 10 μC [3H]-arginine were mixed with 300 μg. E. coli tRNA and 0.1 μmole unlabelled arginine in 1 ml. of ASB. The solution was made 2% with potassium acetate pH 5.0, three volumes of ethanol at -20°C added and the mixture left for 2 hr at -20°C. The resulting precipitate was collected by centrifugation; dissolved in 1 ml. 0.01 M-sodium acetate, 0.002 M EDTA pH 5.5, diluted with 9 ml. 0.01 M ammonium formate pH 5.0 and chromatographed on DEAE cellulose (formate) as for Fig. 2 (a). Of the initial 2.2 × 10^6 counts/min. 2.52 × 10^6 counts/min. were recovered in the wash, and 5.04 × 10^6 counts/min. in the peaks shown in the elution profile.
to elute the main arginyl oligonucleotide fragment(s) produced after T1 RNase digestion of BHK-tRNA (see Fig. 2). The proportion of the contaminants increases in older samples.

[3H]-arginine was also mixed with unlabeled arginine and E. coli tRNA in conditions similar to those used to aminoacylate tRNA in the previous work. The mixture was precipitated with ethanol and redissolved. Fig. 1b gives the radioactive profile obtained after DEAE cellulose chromatography of this material. The contaminants survived the ethanol precipitation and eluted in the same position as before. This material constituted 0.23% of the initial radioactivity. The level of contamination in the [14C]-arginine was very much lower.

In the series of experiments to be described, the tRNA was re-isolated on DEAE cellulose after aminoacylation, thereby eliminating the contaminants, which eluted well ahead of the aminoacyl tRNA. In addition, the aminoacyl-tRNA bond was stabilized by N-acetylation. This overcomes the problem of extensive discharging of the tRNA during T1 RNase digestion which the previous workers had encountered.

Table 1. Extent of tRNA aminoacylation

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Source of aminoacyl tRNA, and source</th>
<th>Specific radioactivity (counts/min./mg.)</th>
<th>Percentage aminoacylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>[14C]-Arginyl tRNA from BHK cells</td>
<td>5.0 x 10^6</td>
<td>4.3</td>
</tr>
<tr>
<td>II</td>
<td>[14C]-Arginyl tRNA from herpes infected BHK cells</td>
<td>3.8 x 10^6</td>
<td>3.2</td>
</tr>
<tr>
<td>III</td>
<td>[14C]-Arginyl tRNA from herpes infected BHK cells</td>
<td>3.5 x 10^5</td>
<td>3.0</td>
</tr>
<tr>
<td>IV</td>
<td>[3H]-Arginyl tRNA from BHK cells</td>
<td>5.1 x 10^6</td>
<td>4.3</td>
</tr>
<tr>
<td>V</td>
<td>[3H]-Arginyl tRNA from herpes infected BHK cells</td>
<td>3.7 x 10^6</td>
<td>3.1</td>
</tr>
<tr>
<td>VI</td>
<td>[3H]-Leucyl tRNA from E. coli cells</td>
<td>4.06 x 10^6</td>
<td>2.2</td>
</tr>
<tr>
<td>VII</td>
<td>[3H]-Leucyl tRNA from T4 infected E. coli cells</td>
<td>4.05 x 10^6</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Extent of aminoacylation

The specific radioactivity and the extent of aminoacylation of the various N-acetyl-aminoacyl tRNAs, as used in this study, are shown in Table 1.

These tRNA preparations were, however, contaminated with RNA species other than tRNA, as judged by Sephadex G-100 chromatography. In tRNA from BHK cells the contaminants constituted about 30 to 35%, and in tRNA from herpes-infected cells about 45%, of the total RNA. When these figures are taken into account, it is evident that 6.2 to 6.7% of BHK tRNA, and 5.5 to 5.8% of tRNA from infected cells, became charged with arginine. These figures are considered to represent a high degree of aminoacylation of the tRNA species involved. The tRNA from E. coli was also contaminated with non-tRNA nucleic acid, but differences between material from T4 infected cells and uninfected cells was not investigated. These high levels of contamination were tolerated in order to minimize the possibility that virus-coded tRNAs might be lost during isolation. A synthetase preparation which was not highly purified was used for similar reasons. The specific radioactivity remained constant during the N-acetylation of the aminoacyl tRNAs.
Arginyl transfer RNA in herpes virus infected cells

Fig. 2. The elution profiles, from DEAE-cellulose (formate) columns (1 x 20 cm) of N-acetyl [14C]-arginyl tRNAs digested with ribonuclease T.1 (a) from BHK cells (no. I, Table I); (b) from herpes infected BHK cells (no. II, Table I); (c) from herpes infected BHK cells but aminoacylated with the synthetase from BHK cells (no. III, Table I) and (d) from herpes infected BHK cells but 'unloaded' prior to aminoacylation.
Ribonuclease T1 digests of N-acetyl [14C]-arginyl tRNA and chromatography of the resulting fragments

As can be seen from Fig. 2a, b, identical profiles were obtained after DEAE cellulose chromatography of N-acetyl [14C]-arginyl tRNA digests irrespective of the source of the tRNA. The same result was obtained with N-acetyl [14C]-arginyl tRNA prepared by aminoacylating tRNA from herpes infected BHK cells, with the synthetase from uninfected cells (Figure 2c). Stripping the tRNA of amino acid by treatment with 1.8 M-tris pH 8.0 for 1 hr at 37°, prior to aminoacylation and N-acetylation, and subsequent ribonuclease T1 digestion of the resulting N-acetyl [14C]-arginyl tRNA, gave exactly the same result (Fig. 2d). The non-enzymic breakdown of N-acetyl [14C]-arginyl tRNA was always low (less than 15 %) and gave rise to [14C]-arginine and N-acetyl [14C]-arginine only. The production of these compounds was only slightly increased in the presence of T1 ribonuclease and they were recovered in the DEAE-cellulose column washes. These washes were checked by paper chromatography (in isobutyric acid/water/concentrated ammonia 66/33/1 by volume) for the presence of fragments, such as CpCpA-arginine [14C], which would bear no net charge and which might not be absorbed by the DEAE-cellulose column. None were found.

These experiments were repeated several times, and no significant variation in the profiles was detected. The distribution of radioactivity among the peaks was also very similar in all experiments (see Table 2).

Table 2. The distribution of radioactivity among the products of ribonuclease digestion of N-acetyl [14C]-tRNAs from different sources.

<table>
<thead>
<tr>
<th>Source of tRNA and synthetase</th>
<th>Peaks eluted from DEAE-cellulose as in Fig. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>BHK tRNA-BHK synthetase</td>
<td>*85.3</td>
</tr>
<tr>
<td>Herpes/BHK tRNA-herpes/BHK synthetase</td>
<td>*83.2</td>
</tr>
<tr>
<td>Herpes/BHK tRNA-BHK synthetase</td>
<td>82.3</td>
</tr>
</tbody>
</table>

* Average of four determinations. The upper and lower limit for each peak is shown in parenthesis.

Hybridization studies

Hybridizations were carried out using tRNA charged with a radioactive amino acid as a specific label and the aminoacyl-tRNA bond, which is unstable at hybridization temperatures, was protected by N-acetylation as described in Methods section. After 2 hr at 70° in 2 x SSC, pH 5.0, all the N-acetyl aminoacyl tRNAs used in this study remained more than 95 % unhydrolyzed.

Weiss et al. (1968) and Daniel, Sarid & Littauer (1970) have shown that when T4 phages infect E. coli cells, at least one new species of leucyl-tRNA appears which can hybridize to T4 DNA. This system was used to ensure that the hybridization technique described above would work in our hands. Transfer RNA from T4 infected E. coli charged with [3H]-leucine using synthetase from uninfected E. coli, was N-acetylated as described. Fig. 4a shows that this material hybridized to T4 DNA but not to herpes DNA. From the double reciprocal plot (Fig. 3b) it can be seen that at saturation 1250 counts/min. of [3H] would be bound to 25 μg. of T4 DNA. Since the efficiency of counting [3H] on the nitro-cellulose filters was 5 %, this represents 2.5 x 10⁴ disintegrations/min. bound. The specific radioactivity of [3H]-leucine
used to load the tRNA was $2 \times 10^4 \mu C/\mu$ mole; i.e. $4.4 \times 10^{10}$ disintegrations min/\(\mu\)mole, hence $(2.5 \times 10^9/4.4 \times 10^{10}) \mu$ moles of $[^3H]$-leucine would be bound at saturation. If it is assumed that the leucyl tRNA was fully charged, the same number of $\mu$ moles of leucyl tRNA would be bound. The mol. wt of T4 DNA is $1.5 \times 10^8$ (Rosenbloom & Cox, 1966),

Therefore a filter with 25 $\mu$g. DNA would contain $25/(1.5 \times 10^8)$ $\mu$ moles equal to $1.6 \times 10^{-7}$ $\mu$ moles of T4 DNA. Thus the number of phage cistrons detected with leucyl tRNA harvested at 35 min. post-infection was $2.5 \times 10^4 \times 10^{-7}/4.4 \times 10^{10} \times 1.6 = 3.4$, or about 3 to 4.

Fig. 4a and b shows the results obtained after hybridizing N-acetyl $[^14C]$-arginyl tRNAs prepared from either uninfected BHK cells, or BHK cells infected with herpes simplex virus, to herpes DNA and T4 DNA. The range of RNA:DNA ratios used was similar to that in the T4 experiment of Fig. 3. The radioactivity bound to filters containing herpes DNA was barely higher than those with T4 DNA or no DNA, and no significant difference between
tRNA from infected or uninfected cells could be detected. The mol. wt of herpes DNA is between $0.7 \times 10^8$ and $10^8$ (Russell & Crawford, 1963), the specific radioactivity of [$^{14}$C]-arginine used was $300 \mu C/\mu mole$ and the efficiency of counting of [$^{14}$C] on nitrocellulose membrane filters was 50%. Assuming the arginyl tRNA to be fully charged, it can then be calculated that 75 $\mu g.$ of herpes DNA would bind between 250 and 350 counts/min. at saturation, if one cistron for arginyl tRNA were present.

Table 3 shows experiments where the sensitivity of the system was upgraded either by using more DNA per filter or by using [$^3$H]-arginine of high specific activity (12,000 $\mu C/\mu mole$) to acylate the tRNA. In this case between 660 and 940 counts/min. would have been expected to bind to each filter. It is clear that filters with herpes DNA bound few, if any, counts above the control filters and no significant difference was observed between tRNA from infected or uninfected cells.

**DISCUSSION**

Analyses of the oligonucleotide fragments produced by the ribonuclease T1 digestion of $N$-acetyl [$^{14}$C]-arginyI tRNA, prepared using extracts from both herpes infected, and uninfected, BHK cells, failed to provide evidence (qualitative or quantitative) for the existence of virus specified arginyl tRNA(s).

This experimental technique might fail to distinguish between a virus specified tRNA and a host tRNA if they yielded identical aminoacyloligonucleotide fragments. However, even in this case a change in the relative amounts of the fragments might be expected. Since the distribution of radioactivity among the peaks was very similar in all experiments (Table 2), it would have to be argued that the production of any virus specific oligonucleotide fragment had been balanced by a reduction in the corresponding host tRNA fragment. This seems
unlikely. Alternatively, four virus specific fragments might have been produced in the same relative proportions as those from the host tRNA, thereby yielding the same overall pattern. This seems even more unlikely.

Table 3. The hybridization of N-acetyl [3H]-arginyl tRNA and N-acetyl [14C]-arginyl t-RNA prepared from herpes infected and uninfected BHK cells, to herpes and T4 DNA

<table>
<thead>
<tr>
<th>Source of labelled tRNA</th>
<th>DNA on filter</th>
<th>Counts/min. bound above T4 blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes infected BHK cells</td>
<td>[14C]</td>
<td></td>
</tr>
<tr>
<td>200 μg.</td>
<td>995</td>
<td>109</td>
</tr>
<tr>
<td>T4</td>
<td>873</td>
<td></td>
</tr>
<tr>
<td>200 μg.</td>
<td>891</td>
<td></td>
</tr>
<tr>
<td>Herpes infected BHK cells</td>
<td>[3H]</td>
<td></td>
</tr>
<tr>
<td>50 μg.</td>
<td>1545</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1556</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1414</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1549</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1419</td>
<td></td>
</tr>
<tr>
<td>50 μg.</td>
<td>1687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1553</td>
<td></td>
</tr>
<tr>
<td>Uninfected BHK cells</td>
<td>[14C]</td>
<td></td>
</tr>
<tr>
<td>200 μg.</td>
<td>798</td>
<td>65</td>
</tr>
<tr>
<td>T4</td>
<td>738</td>
<td></td>
</tr>
<tr>
<td>200 μg.</td>
<td>695</td>
<td></td>
</tr>
<tr>
<td>Uninfected BHK cells</td>
<td>[3H]</td>
<td></td>
</tr>
<tr>
<td>50 μg.</td>
<td>1864</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>1615</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1752</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1711</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1670</td>
<td></td>
</tr>
<tr>
<td>50 μg.</td>
<td>1671</td>
<td></td>
</tr>
</tbody>
</table>

880 μg N-acetyl [14C]-arginy1 tRNA from uninfected BHK cells (tRNA, Table 1) or 980 μg. of N-acetyl [14C]-arginy1 tRNA from herpes infected cells (tRNA II, Table 1) were hybridized to 200 μg. herpes or T4 DNA immobilized on 40 mm. nitro cellulose membrane filters immersed in 2 ml. of 2 × SSC pH 5.0. The N-acetyl [3H]-arginyl tRNA from uninfected cells (q80 μg.; tRNA IV, Table 1; 2.5 × 10^6 counts/min. in total) and from herpes infected cells (670 μg.; tRNA V, Table 1; 2.4 × 10^6 counts/min. in total) were hybridized to 50 μg. of herpes or T4 DNA immobilized on 24 mm filters immersed in 2 ml. of 2 × SSC pH 5.0. Hybridization and screening were carried out as described and results are expressed as counts/min. above background.

Our reconstruction experiments demonstrate that the peaks described as virus specific arginyl oligonucleotides by Subak-Sharpe, et al. (1966) were probably due to the presence of contaminants in the radioactive arginine used.

Previous studies also showed that [32P]-labelled 4s RNA from herpes infected BHK cells hybridized to herpes DNA (Subak-Sharpe & Hay, 1965). However, it is difficult to be sure that such preparations are not contaminated with RNA other than tRNA which are capable of extensive hybridization to homologous DNA. Such contaminants have been identified in other systems; (uninfected E. coli, Wilkie, unpublished observations; 80 infected coli, Landy et al. 1967). The specific method of tRNA hybridization used in this study overcomes this problem.

Fig. 3 shows that the method of hybridization utilizing N-acetyl aminoacyl tRNA was successful in our hands. Assuming that the leucyl tRNA was fully charged, we could calculate that T4 DNA contains 3 to 4 cistrons for leucyl tRNA. This must be regarded as a minimum estimate, since the presence of any unlabelled leucyl specific tRNAs would reduce the specific radioactivity of the bound tRNA. The presence of more than one cistron for leucyl tRNA in T4 DNA is in agreement with the results of Waters & Novelli (1967) who reported.
the appearance of at least two new peaks of leucyl tRNA upon reverse phase column chromatography of tRNA from T2 infected E. coli.

The results presented in Fig. 4 and Table 3 show that we failed to obtain evidence for herpes virus specified arginyl tRNA in experiments where the number of counts/min. expected to bind to one cistron, at saturation, ranged from about 250 to 940 counts/min. Again, incomplete aminoacylation, or discharging during hybridization and screening would result in a reduction of the expected number of counts/min. However, as indicated above, the extent of aminoacylation seemed high and only a small degree of uncharging could have occurred during the hybridization and screening. Of course, if the sites for tRNA on the herpes DNA were not fully saturated, a smaller number of counts/min. would be expected. However, the high excess of RNA over DNA used makes failure to saturate unlikely. In the experiment presented in Table 3, a 13-fold excess of RNA over DNA was used as compared with the $1.5:1$ ratio necessary to achieve $75\%$ saturation of the multiple leucyl tRNA sites on T4 DNA (Fig. 3).

It is theoretically possible that virus specified tRNA was not detected by either of the methods described above because the tRNA was (a) lost during isolation, (b) not aminoacylated with labelled amino acid under the conditions used, or (c) deacylated during ribonuclease T1 digestion. However, every precaution was taken to extract all tRNAs, including those bound to polysomes, and since there was relatively little loss of label during digestion, possibilities (a) and (c) seem unlikely. Although aminoacylation appeared to be extensive, possibility (b) cannot be ruled out.

These findings are in agreement with the results of Morris et al. (1969, 1970) who purified generally labelled arginyl- and seryl-specific tRNA from herpes infected HEp-2 cells by chromatography on benzoylated DEAE-cellulose, and subsequently showed that this material did not hybridize to herpes DNA. In addition, the hybridization of generally labelled 4s RNA from infected cells to virus DNA could be almost completely eliminated by competition with high molecular weight nuclear RNA.

The excellent technical assistance of D. Joicey and A. Revill is gratefully acknowledged. We also wish to thank Dr. U. Z. Littauer for information on the N-acetylation and hybridization of aminoacyl tRNA prior to its publication.

REFERENCES


Arginyl transfer RNA in herpes virus infected cells


SUBAK-SHARPE, H. & HAY, J. (1965). An animal virus with DNA of high guanine + cytosine content which codes for sRNA. *Journal of Molecular Biology* 12, 924.


(Received 7 May 1971)