Variations in Hybridization of RNA from Different Mouse Tissues and Embryos to Endogenous C-type Virus DNA Transcripts

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SUMMARY

Several adult tissues, newborns, and embryos of uninfected BALB/c mice were analysed for RNA complementary to \[^3\text{H}]\)-DNA transcripts synthesized from an endogenous type-C virus of BALB/c 3T3. The technique of RNA:DNA hybridization was used and the extent of hybridization was measured by the use of a single-strand-specific nuclease (S-1), purified from Aspergillus oryzae.

Virus-specific RNA was detected in all adult and embryonic tissues tested. However, the RNA extracted from tissues having higher proliferative activity, such as spleen, small intestine, uterus and embryos, hybridize the \[^3\text{H}]\)-DNA probe to a greater extent than the RNA from tissues with low proliferative activity, such as kidney and liver. These observations add further support to the view that the repression of the virus genome in normal cells is not complete, and suggest the existence of a correlation between a qualitative or quantitative change in the endogenous C-type virus genome transcription pattern and cell proliferation.

INTRODUCTION

Several lines of experimental evidence suggest that the cells of most or all vertebrate species contain endogenous C-type RNA virus genomes. For example, uninfected cells of clonal origin, in culture, release C-type RNA viruses either spontaneously (Aaronson, Hartley & Todaro, 1969; Rowe et al. 1971; Todaro, 1972) or after treatment with certain inducing agents such as halogenated pyrimidines (Aaronson, Todaro & Scolnick, 1971; Lowy et al. 1971). It has been shown that the type-C RNA virus-specific sequences exist in high mol. wt. cellular DNA of all normal vertebrate cells tested (Baluda, 1972; Varmus et al. 1972; Benveniste et al. 1974) and such virus genomes are partially expressed in uninfected adult and embryonic tissues (Benveniste et al. 1973; Parks et al. 1973). Furthermore, several recent studies suggest that genomes of more than one distinct host-range class of endogenous virus, which show only partial nucleic-acid homology, are present in a single cell (Benveniste, Lieber & Todaro, 1974).

It has been suggested that the C-type RNA virus genome constitutes an integral part of the natural genetic information of all vertebrate cells and such endogenous virus genomes play a role in normal vertebrate development (Todaro & Huebner, 1972). The presence of high titres of group-specific (gs) antigen (Huebner et al. 1970) and RNA, specific to C-type virus genome in uninfected early embryonic cells (Hayward & Hanafusa, 1973) as well as in adult tissues (Benveniste et al. 1973, 1974; Parks et al. 1973) support the above concept. However, the state of the endogenous virus information and its natural role in uninfected cells are still unclear.
The present study was initiated to examine various adult tissues, newborns and embryos of uninfected BALB/c mice for RNA complementary to \[^{3}H\]-DNA transcripts, synthesized from an endogenous C-type virus of BALB/c 3T3. The endogenous reverse transcriptase reaction was used for synthesizing \[^{3}H\]-DNA copy of the virus genome and the hybridization was measured by the use of a single-strand-specific nuclease, purified from *Aspergillus oryzae*.

**METHODS**

**Tissues.** Livers, kidneys, spleens, uteri and small intestines from BALB/c mice (12-week old, Jackson Lab.) were collected in ice-cold phosphate-buffered saline (PBS), immediately after the animals were killed. The tissues were washed twice in cold PBS and stored at \(-20^\circ C\), until used.

**Isolation of embryos.** Eight- to twelve-week-old BALB/c female mice were mated and the pregnant females were identified on the next day by the presence of vaginal plugs. Counting this day as day one, batches of female mice were killed on the 14th, 16th and 18th days of pregnancy and their embryos were collected, washed with PBS and stored at \(-20^\circ C\). Newborn mice were collected immediately after birth.

**Cells.** S\(_2\)C\(_3\) cells (a gift from Dr R. E. Benveniste, NIH, Bethesda) were derived from BALB/c 3T3 clone A31. It is a spontaneously transformed cell line and produces S\(_2\)C\(_3\) virus. The cells were grown in 16 oz prescription bottles, in Dulbecco’s modification of Eagle’s minimal essential medium supplemented with 10% foetal calf serum (Flow Laboratories).

**Extraction of cellular RNA.** Total cellular RNA was isolated from various adult tissues and embryos of BALB/c mice, and S\(_2\)C\(_3\) cells by the modified hot-phenol-SDS (sodium dodecyl sulphate) method (Scherrer, 1970) described by Benveniste & Scolnick (1973).

Frozen tissues were thawed quickly, suspended in ice-cold PBS, minced whenever necessary, washed in PBS and resuspended in 20 vol. of an extraction buffer containing 0.4 M-sodium acetate, pH 5.0; 0.002 M-ethylene diamine-tetra-acetic acid (EDTA); 0.1 M-NaCl and 1% SDS before homogenizing in a Potter-Elvehjem homogenizer. The homogenate was extracted thrice with hot (65°C), neutralized, water-saturated phenol-cresol and with chloroform-isooamyl alcohol (24:1). The RNA was then precipitated with 2 vol. of cold ethanol and treated with DNase I (bovine pancreas, Sigma; 20 \(\mu\)g/ml in a solution containing 0.02 M-tris, pH 7.4; 0.01 M-MgAc and 0.01 M-MaCl) for 90 min. at 37°C. Only DNase, free of contaminating RNAsse A, as judged by its ability to render \[^{3}H\]-polyuridylic acid (poly-A) trichloroacetic acid (TCA) soluble was used. The solution was again phenol-cresol and ether extracted, dialysed exhaustively (against a buffer containing 0.02 M-tris, pH 7.4; 0.02 M-NaCl and 10\(^{-9}\) M-EDTA) and lyophilized. Purified RNA was stored at \(-20^\circ C\), at a concentration of 10 to 20 mg/ml in distilled water. Twenty-three \(E_{260}\) units were taken to represent 1 mg/ml of RNA. The \(E_{260}/E_{280}\) ratio was greater than 2 for all preparations used.

**Synthesis and purification of virus-specific \[^{3}H\]-DNA.** A spontaneously induced endogenous C-type RNA virus, S\(_2\)C\(_3\), collected from supernatant fluids of S\(_2\)C\(_3\) cultures and purified by isopycnic banding on sucrose gradients was a gift from Dr R. E. Benveniste (NIH, Bethesda, Md). S\(_2\)C\(_3\) virus was chosen for this study because it is an endogenous BALB/c virus.

The endogenous reverse transcriptase reaction of Fan & Baltimore (1973) was used, with some modifications, to synthesize \[^{3}H\]-labelled DNA probe, complementary to S\(_2\)C\(_3\) virus RNA. Two ml of reaction mixture containing 0.02 M-tris, pH 8.0; 0.02 M-
C-type virus RNA : DNA hybridization
dithiothreitol (DTT); 0.06 M-NaCl; 6 mM-MgAc; 1 mM each of dATP, dGTP and dCTP; 
$4 \times 10^{-5}$ M-[3H]-TTP (50 Ci/mm, Schwarz/Mann); 0.02% Triton X-100; 9 mM-creatine phosphate; 
100 mg creatine phosphokinase/ml and 60 mg of actinomycin D with purified 
$S_{2}\text{Cl}_{3}$ virus (approx. 0.2 to 0.4 mg virus protein/ml) was incubated for 3 h at 37 °C. At the 
end of incubation SDS and NaOH were added to the reaction mixture to a final concen-
tration of 2% and 0.3 M respectively, heated at 100 °C for 5 min and neutralized. The 
sample was then applied to a Sephadex G-50 column, and fractions containing acid-
precipitable radioactivity were pooled and lyophilized. The lyophilized material was sus-
pended in H$_2$O, adjusted to 0.5 M-NaCl and precipitated with cold ethanol. The precipitate 
was then collected by centrifuging and resuspended at a concentration of 1000 ct/min/µl 
in distilled water. The product was then stored at −20 °C.

_Extraction of unlabelled $S_{2}\text{Cl}_{3}$ virus RNA_. $S_{2}\text{Cl}_{3}$ virus RNA was prepared according 
to the method of Ross, Tronick & Scolnick (1972). Purified viruses were disrupted by 
suspending in TNE buffer (0.01 M-tris, pH 7.5; 0.01 M-NaCl; 0.001 M-EDTA) containing 
1% SDS, extracted three times with water-saturated phenol-cresol and precipitated with 
cold ethanol. The precipitate was then collected by centrifuging, resuspended in TNE 
buffer containing 0.1% SDS and purified by centrifuging through a 5 to 20% sucrose 
gradiant.

_S-1 nuclease_. A single-strand-specific nuclease of _Aspergillus oryzae_ was purified from 
α-amylase (diastase) powder (Sigma Chemical Co.) by DEAE-cellulose and G-75 Sephadex 
chromatography, according to the method described by Sutton (1971) and Ando (1966). 
The enzyme was stored in 50% glycerol at −20 °C.

_Nucleic acid hybridization and analysis_. The nucleic acid hybridization method described 
by Benveniste & Scolnick (1973) was used. Approx. 2000 ct/min (0.1 ng) of single stranded, 
$S_{2}\text{Cl}_{3}$ virus-specific [3H]-DNA product was incubated with increasing amounts of RNA 
for 72 h at 41 °C in 0.2 ml reaction mixture (0.015 M-tris, pH 7.3; 0.15 M-NaCl; 5 × $10^{-4}$ M-
EDTA and 0.1% SDS) containing 38% formamide. Duplicate tubes containing the 
appropriate level of RNA were prepared for each hybridization reaction, one incubated 
at 41 °C and the other was kept at −70 °C for the length of the hybridization. At the end 
of incubation, 2.3 ml of S-1 enzyme solution (0.033 M-NaAc, pH 4.5; 2 × $10^{-4}$ M-ZnSO$_4$; 
0.13 M-NaCl; 30 µg of denatured calf thymus DNA) containing 12 µl of S-1 nuclease was 
added to each tube (frozen and incubated). This amount of S-1 nuclease was found to 
be sufficient to degrade 96 to 98% of the input [3H]-DNA counts even in the presence 
of a large excess of RNA. The tubes were then incubated at 45 °C for 70 min. Hybridization 
was measured as the percentage of input [3H]-DNA counts that remained TCA precipitable 
after S-1 nuclease digestions. The frozen sample was used to determine whether the large 
excess of RNA present in a reaction impeded the degradation of unhybridized [3H]-DNA 
product by the S-1 nuclease. The background counts, remainder after S-1 digestion of the 
control (frozen) reaction (2 to 4% of the input counts), were subtracted from the counts 
obtained from the homologous, incubated (41 °C) reaction, before calculating the hybridi-
ization value for each of the RNA samples used.

**RESULTS**

In order to determine the extent to which the virus [3H]-DNA probe was complementary 
to its RNA template, approx. 2000 ct/min of $S_{2}\text{Cl}_{3}$ [3H]-DNA product was hybridized 
with increasing amounts of unlabelled $S_{2}\text{Cl}_{3}$ 60 to 70S RNA. At saturation, approx. 95% 
of the [3H]-DNA probe was complementary to the virus RNA (data not shown). However,
the proportion of the virus genome (60 to 70S RNA) that is represented in the [3H]-DNA probe was not determined. The digestion of S2Cl₃ [3H]-DNA product with the single-strand-specific S-I nuclease showed it to be almost 98 % single-stranded. Furthermore, it has been shown recently that the [3H]-DNA product synthesized by the endogenous reverse transcriptase reaction, in the presence of actinomycin D is a complete and relatively uniform copy of the entire virus genome (Garapin et al. 1973). Therefore, such a DNA product provides a valid probe for determining the portion of the virus genome that is represented in a given sample of cellular RNA, by molecular hybridization.

The results presented in Fig. 1 show that RNA isolated from S2Cl₃ cells, which was used as a control, hybridizes the S2Cl₃ [3H]-DNA product at a level of approx. 69.5 %. This saturation level of hybridization was achieved with the addition of 50 to 60 µg of RNA in the reaction. The hybridization value for this RNA, obtained in the present experiment was comparable to the value obtained by Benveniste & Scolnick (1973). Fig. 1 also shows that while the kidney and liver tissues of BALB/c mice contain RNAs which hybridize approx. 5 % of the S2Cl₃ [3H]-DNA probe, RNAs from uterine, intestinal and spleenic tissues of the same animals hybridize the probe to a greater extent, at approx. 9 %, 11 % and 18 % levels respectively. The data presented in Fig. 2 show that the RNA from newborn BALB/c mice (whole animal) hybridizes to only 2.5 % of the S2Cl₃ DNA probe, 18-day embryo (whole embryo) to about 6 %, 16-day embryo about 8.5 % and 14-day

Fig. 1. Analysis of hybridization of RNA from various tissues of BALB/c mice, spontaneously transformed, producer BALB/c 3T3 cells (S2Cl₃), and yeast RNA to endogenous C-type virus-specific S2Cl₃ [3H]-DNA transcripts. □ —□, S2Cl₃ cell (control); × — ×, yeast (control); ●— ●, spleen; △—△, intestine; △—△, uterus; ■— ■, liver; ○—○, kidney.
C-type virus RNA: DNA hybridization

Fig. 2. Results of hybridization of RNA from newborns and embryos of BALB/c to S₂Cl₂ virus-specific [³H]-DNA product. • — •, 14-day embryo; ○ — ○, 16-day embryo; △ — △, 18-day embryo; ▲ — ▲, newborn.

embryo yields approx. 7% hybridization level. Although, hybridization reactions with RNA from embryonic tissues and newborn animals could not be brought to the saturation levels, RNA from 14-, 16-, and 18-day embryos hybridized more of the input [³H]-DNA probe than the RNA from newborn animals, at each level of RNA used.

No detectable hybridization of the [³H]-DNA probe could be obtained with increasing amounts of poly-(A) (data not shown). This reaction was included as a control to test the possibility that a portion of the [³H]-DNA probe might have been transcribed from the poly-(A) sequences of the virus RNA (Ross et al. 1972) and hybridized with adenine-rich sequences of any non-virus-specific cell messenger RNA. Neither could detectable hybridization be obtained with increasing amounts of yeast tRNA (Fig. 1). Both hybridization values (with poly-(A) and yeast tRNA) were also calculated upon subtraction of the background counts which remained after S-1 digestion of the control, frozen reactions.

DISCUSSION

The data obtained from the present experiment reveal that various adult tissues, newborns, and embryos of uninfected BALB/c mice, all contain RNA which hybridizes to a portion of the S₂Cl₂ [³H]-DNA probe. In addition, the data also show that RNA isolated from embryonic, splenic, intestinal, and uterine tissues hybridizes the [³H]-DNA product to a greater extent than the RNA from newborns, adult livers and kidneys of the same animals. It is unlikely that such hybridizable RNAs are complementary to cellular sequences that might have been represented in the [³H]-DNA probe, since almost all (95 to 98%) of the input [³H]-DNA counts in a reaction become resistant to S-1 nuclease digestion, when hybridized with purified 60 to 70S RNA of S₂Cl₂ virus. The RNA from various tissues, which hybridizes with the [³H]-DNA probe is, therefore, virus-specific. However, from the data obtained in the present experiment it is not possible to distinguish between a qualitative (new sequences) and quantitative (amount) difference in the virus genome.
transcription pattern in various tissues because, the proportion of the S_{2}Cl_{3} virus genome that is represented in the $^{3}H$-DNA transcript is not determined, the percentage of virus-specific RNA in different RNA samples may vary, and because the saturation hybridization levels for RNAs from several tissues are not achieved.

Partial expression of the murine type-C virus genome in uninfected mouse cells, in vivo and in vitro has been detected previously by other investigators. Using a highly sensitive radioimmunoassay, specific for the C-type virus gs antigen, Parks et al. (1973) detected antigenic reactivity in various tissues of weanling and young adult mice (C57L/J and BALB/c). The complement fixation (CF) tests for gs antigen carried out by Huebner et al. (1970) showed high titres of gs antigen in uninfected mouse embryos. Using indirect immuno-autoradiography, Abelev & Elgort (1970) showed the presence of MuLV gs antigen in adult and embryonic tissues of low leukaemic strains of mice. Benveniste et al. (1973) detected virus-specific RNA in uninfected BALB/c 3T_{3} cells by nucleic acid hybridization. The above observations and the data obtained in the present study, add further support to the view that the repression of the endogenous virus genome in normal cells is not complete and at least a portion of it is widely expressed.

It is interesting to note that tissues, such as spleen, intestine, uterus and embryo, which contain RNA that hybridizes more of the virus $^{3}H$-DNA probe than the RNA from kidney and liver (Fig. 1 and 2) also show higher proliferative activity than that of the latter tissues. A comparable study by Benveniste et al. (1974) with various baboon tissues shows that the RNA from baboon liver hybridizes at the saturating level of approx. 10% of the baboon C-type RNA virus $^{3}H$-DNA probe but RNAs from lung, spleenic, testicular and placental tissues of the same animals show approx. 35, 40, 55 and 70% levels, respectively. In several other studies (Huebner et al. 1971; Abelev & Elgort, 1970) the expression of gs antigen has been found to be highly pronounced in early whole embryo tissues and in reproductive, intestinal and haematopietic tissues (all show continuous cell proliferation) but the expression of such antigen is almost undetectable in skeletal and heart muscle (which show low proliferative activity). Furthermore, it has been shown (Vincent, Mukherjee & Mobry, unpublished data), by employing molecular hybridization, that adult mouse liver exhibits a minimal rate of DNA synthesis and a low level of virus genome transcription. But with a significant elevation of DNA synthesis (and therefore cell division) in regenerating liver, after partial hepatectomy, there is a concomitant increase in the content of virus-specific RNA in the hepatocytes.

The observations discussed above suggested the existence of a correlation between a qualitative or quantitative change in the endogenous C-type virus genome transcription pattern and cell proliferation. Based on the observation that early mouse embryo cells and adult tissues which are subject to continuous cell replication contain high titres of gs antigen of the C-type RNA virus and such gs antigen expression is relatively undetectable in the non-proliferating tissues, Huebner et al. (1971) suggested that the virus genome may play a determining role in regulating cell proliferation. This is a very interesting concept, and if it can be determined that partial virus genome expression is indeed involved in the process of cell proliferation, it will offer a natural role for the endogenous C-type virus genomes in normal vertebrate cells.

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