Changes in Transcription of Endogenous Type-C Virus Genome During Mouse Liver Regeneration

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SUMMARY

In the present study the extent of endogenous, type-C virus genome transcription in normal and regenerating mouse liver was analysed by using the technique of nucleic acid hybridization. The RNA preparations from regenerating liver tissues collected at various intervals following partial hepatectomy, and from normal liver samples of BALB/c mice, were hybridized to 3H-DNA complementary to 60 to 70S RNA of an endogenous, N-tropic virus, released spontaneously from BALB/c mouse cells in culture. Although partial transcription of the endogenous virus genome can be clearly detected in normal liver, a significant increase in the level of virus-specific RNA synthesis in the regenerating liver, in comparison to normal liver, is apparent, following partial hepatectomy. This increase in virus-specific RNA synthesis attains its highest level just before the level of DNA synthesis in the regenerating liver reaches its maximum. These observations may indicate a qualitative or quantitative change in the endogenous type-C virus genome transcription pattern in hepatocytes, in response to partial hepatectomy and suggest that this change in the transcription pattern and the initiation of cell proliferation, in regenerating livers, are probably sequential and related events.

INTRODUCTION

The presence of genetic information for type-C RNA viruses in the chromosomal DNA of many mammalian species has been well established (Gelb et al. 1973; Benveniste & Todaro, 1974; Chattopadhyay et al. 1974; Scolnick et al. 1974; Todaro et al. 1974). Although, the natural role of the endogenous virus genomes in uninfected cells is still unclear, the synthesis of virus-specific RNA and virus gene products in a wide variety of cells have been detected (Abelev & Elgort, 1970; Benveniste et al. 1973; Parks et al. 1973; Stephenson & Aaronson, 1973; Sherr, Benveniste & Todaro, 1974; Fan & Besmer, 1975). Huebner et al. (1970, 1971) have reported the expression of group-specific antigen (gs) of the C-type RNA tumour virus in whole embryo tissues and in rapidly replicating postnatal tissues of several mouse strains. Our previous study (Mukherjee & Mobry, 1975) has shown that early mouse embryos and adult tissues having high proliferative activity contain RNA molecules which hybridize with the 3H-DNA copies of an endogenous type-C virus genome to a greater extent than the RNAs from tissues with low proliferative activity. These observations clearly indicate that at least a portion of the endogenous virus genome is widely expressed in many types of uninfected cells and suggest further the existence of a correlation between a change in the virus genome transcription pattern and cell proliferation.
It has been shown that the cells of adult mouse liver, with a low mitotic rate, suddenly begin rapid proliferation following partial hepatectomy and the synthesis of new RNA molecules, not present in adult liver, commences during liver regeneration (Church & McCarthy, 1967a). The cells of regenerating liver have also been found to share antigens with embryonic and neoplastic cells, all of which undergo rapid proliferation (Hellstrom, Hellstrom & Nishioka, 1975). In the context of the observations mentioned above, the question may be asked whether re-activation of certain endogenous type-C virus-genes, which are repressed in adult liver, takes place during liver regeneration. The present study is an attempt to answer this question by hybridizing cytoplasmic RNA from normal and regenerating livers of BALB/c mice to single-stranded 3H-DNA copies of an endogenous, N-tropic BALB/c virus genome.

**METHODS**

*Partial hepatectomy.* Ten-week old female BALB/c mice (Jackson Lab.) were anaesthetized with Avertin (Winthrop Lab., N.Y.; 200 mg 2-2-2 tribromoethanol dissolved in 1.0 ml of tert-amyl alcohol and diluted 1 part in 25 with sterile saline; 0.02 ml/g body weight, injected intraperitoneally). A two-third hepatectomy was performed by the removal of the median and the left lateral liver lobes, leaving within the peritoneum the right lateral and the small caudate lobes. The abdomen was then closed and the remaining liver was allowed to undergo regeneration. Regenerated liver tissues were collected in cold (4 °C) phosphate-buffered saline (PBS), from animals killed at 12 h, 20 h and 36 h, following hepatectomy and stored at −70 °C until use. All operations were timed so that harvesting of tissues took place between 8 and 10 a.m. in order to minimize variations caused by the circadian periodicity of mitosis (Bucher, 1963). The same procedure was also used to prepare liver tissues from sham-operated animals.

*DNA synthesis in regenerating liver.* In order to measure the extent of DNA synthesis during liver regeneration, hepatectomized mice were injected intraperitoneally (i.p.) with 1.5 μCi of 3H-thymidine (750 Ci/mmol, New England Nuclear Corp.) per gram body weight 1 h prior to sacrifice at 12, 18, 24, 30, 36 h post-hepatectomy. The liver tissues were then collected, washed with PBS, and stored at −70 °C until use. Livers from sham-operated animals were also processed and stored the same way.

DNA was extracted from each sample (average 0.6 g tissue) by a modification of the procedure of Marmur (1961). Finely minced tissues were homogenized in 10 to 20 ml of lysing solution containing 0.05 M-tris-HCl (pH 7.4), 5 mM-Mg acetate, 0.04 M-NaCl; the nuclei thus released were then pelleted at 10000 g for 5 min. The nuclei were thoroughly re-suspended in 5 ml TNE buffer (0.01 M-tris-HCl; pH 7.5, 0.01 M-NaCl, 1 mM-EDTA) and lysed by the addition of sodium dodecyl sulphate (SDS) to a final concentration of 1 %. The sample was then extracted four times with re-distilled, water-saturated phenol. The de-proteinized DNA was precipitated with ethanol and collected by centrifuging at 10000 g for 30 min. The DNA pellets were then washed successively in three changes of 15 ml each of ice-cold 10 % trichloroacetic acid (TCA), and one wash with 10 % TCA kept at room temperature. They were then resuspended and dissolved in 1 ml of 10 % TCA by boiling for 30 min. The remaining precipitate was removed by centrifugation, and samples of the supernatant fluid were assayed for radioactivity and DNA content. DNA estimations were performed by the diphenylamine reaction (Shatkin, 1969); scintillation counting was done using 10 ml of perchloric acid (PCA) solubilizer (Amersham-Searle).

*Extraction of cytoplasmic RNA.* Cytoplasmic RNA was extracted from liver tissues by the modified phenol-SDS method, described by Fan & Baltimore (1973). Tissues were minced in
ice-cold PBS and washed twice before homogenizing in a Potter–Elvehjem homogenizer, in 20 vol. of an extraction buffer containing 0.01 M-NaCl, 1.5 mM-MgCl₂, 0.01 M-tris-HCl (pH 7.4) and 1% Triton X-100. The nuclei were pelleted at 10000 g for 5 min at 4 °C, and the supernatant fluid was adjusted to a final concentration of 0.4 M-NaCl, 0.01 M-EDTA and 1% SDS. The supernatant fluid was extracted three to four times with an equal volume of neutralized, water saturated, re-distilled phenol and chloroform-isooamyl alcohol (24:1). The RNA was precipitated with 2 vol. of cold ethanol, collected by centrifugation at 10000 g for 1 h, treated with DNase, dialysed exhaustively against a buffer containing 0.001 M-tris-HCl (pH 7.4) and 10⁻⁴ M-EDTA and lyophilized. Purified RNA was stored at −20 °C, at a concentration of 10 to 15 mg/ml in a final buffer containing 0.01 M-tris-HCl (pH 7.4) and 10⁻⁸ M-EDTA. Twenty-three E₂₆₀ units were taken to represent 1 mg/ml of RNA. The E₂₆₀/E₂₈₀ ratio was greater than 2 for all preparations used.

**Synthesis and purification of virus specific ³H-DNA.** Endogenous, N-tropic BALB/c viruses (S₂Cl₂) were collected from supernatant fluids of S₂Cl₂ cell cultures (derived from BALB/c 3T3; Todaro, 1972) and purified by isopycnic banding on sucrose gradients. The endogenous reverse transcriptase reaction of Fan & Baltimore (1973) was used, with some modifications, to synthesize ³H-labelled DNA probe complementary to S₂Cl₂ virus RNA. Two ml of the reaction mixture containing 0.02 M-tris, pH 8.0, 0.02 M-dithiothreitol, 0.06 M-NaCl, 6 mM-Mg acetate, 1 mM each of dATP, dGTP and dCTP, 4 × 10⁻⁵ M-³H-TTP (50 Ci/mm, Schwarz/Mann), 0.01% Triton X-100, 9 mM-creatine phosphate, 100 mg creatine phosphokinase/ml and 60 mg of actinomycin D with purified S₂Cl₂ virus (approx. 0.2 to 0.4 mg virus protein/ml) were incubated for 3 h at 37 °C. At the end of the incubation, SDS and NaOH were added to the reaction mixture to a final concentration 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 suspended in H₂O, adjusted to 0.5 M-NaCl, and precipitated with cold ethanol. The precipitate was then collected by centrifugation and resuspended at a concentration of 1000 cts/min/μl in distilled water. The product was then stored at −20 °C.

**Extraction of unlabelled S₂Cl₂ virus RNA.** S₂Cl₂ virus RNA was prepared according to the method of Ross, Tronick & Scolnick (1972). Purified viruses were disrupted by suspending in TNE buffer containing 1% SDS, extracted three times with water-saturated phenol-cresol and precipitated with cold ethanol. The precipitate was then collected by centrifugation, resuspended in TNE buffer containing 0.1% SDS and purified by centrifugation through a 5 to 20% sucrose gradient.

**S₁ 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. The digestion of ³H-labelled native and heat-denatured φX 174 RF DNA with the nuclease preparation showed the high single-strand specificity of this enzyme.

**Nucleic acid hybridization and analysis.** A modification of the nucleic acid hybridization method described by Benveniste & Scolnick (1973) was used. Approx. 2000 cts/min (0.1 ng) of single-stranded, S₂Cl₂ virus-specific ³H-DNA product was incubated with increasing amounts of RNA for 72 h at 45 °C in 0.2 ml reaction mixtures containing 0.015 M-tris, pH 7.3, 0.3 M-NaCl, 5 × 10⁻⁴ M-EDTA, 0.1% SDS and 35% formamide. Duplicate tubes were prepared for hybridization reactions containing a large excess of RNA; one was incubated at 45 °C and the other kept at −70 °C for the length of the hybridization. At the end of 72 h,
Fig. 1. The extent of DNA synthesis by hepatocytes at various intervals after partial hepatectomy. The sp. act. of the extracted DNA was determined following a 1 h pulse of ³H-thymidine prior to sacrificing the animals (BALB/c) at the indicated times after partial hepatectomy.

2.3 ml of S₁ enzyme solution (0.033 M-Na Acetate, pH 4.5, 2 × 10⁻⁴ M-ZnSO₄, 0.13 M-NaCl, 30 μg of denatured calf thymus DNA) containing 12 μl of S₁ nuclease was added to each tube (both frozen and incubated). This amount of S₁ 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 S₁ nuclease digestion. 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 S₁ nuclease. The background counts, remaining after S₁ digestion of the frozen reactions (2 to 4 % of the input counts), were subtracted from the counts obtained from the incubated (45 °C) reactions before calculating the hybridization values for each of the RNA samples used.

Tm determination of the RNA-DNA hybrids. Approx. 5000 ct/min of single-stranded S₂Cl₅ virus-specific ³H-DNA product was incubated separately with saturating levels of S₂Cl₅ virus 60 to 70 S RNA and RNA from 20 h regenerating liver, in a 0.4 ml incubation mixture for 72 h. The hybridization method was the same as described previously. At the end of incubation at 45 °C, 40 μl samples of each incubation mixture were expelled into 2.3 ml of S₁ enzyme mixture, without the S₁, heated for 5 min at various temperatures and chilled rapidly at 4 °C. S₁ nuclease was then added and the digestions were carried out at 45 °C for 70 min. The samples were then assayed for the amount of acid-precipitable hybrid present.

RESULTS

DNA synthesis in regenerating livers

The data presented in Fig. 1 show that a significant increase of DNA synthesis in liver cells occurs at about 18 h after partial hepatectomy, reaching a maximum at about 24 h. This is followed by a gradual decline and at 36 h the extent of DNA synthesis returns to approx. the same level seen at 12 h post-hepatectomy. This sudden burst of DNA synthesis in regenerating livers shows that a large number of hepatocytes undergo rapid proliferation in response to partial hepatectomy.
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Fig. 2. Hybridization of cytoplasmic RNA isolated from regenerating livers, livers of sham-operated and control adult BALB/c mice, with S<sub>2</sub>C<sub>13</sub> virus specific <sup>3</sup>H-DNA. The arrows indicate the respective saturation hybridization values for each of the five samples. The level obtained using RNA extracted from S<sub>2</sub>C<sub>13</sub> producing BALB/c cells (3T3, clone A31) was 69 %. (a) RNA from 12 h post-hepatectomy; (b) RNA from 20 h post-hepatectomy; (c) RNA from 36 h post-hepatectomy; (d) RNA from: ••••, control and ○---○, sham-operated mouse livers.

Characterization of virus-specific <sup>3</sup>H-DNA probe

In order to determine the extent to which the virus <sup>3</sup>H-DNA probe was complementary to its RNA template, approx. 2000 cpm of <sup>3</sup>H-DNA product (S<sub>2</sub>C<sub>13</sub> virus) was hybridized with increasing amounts of unlabelled S<sub>2</sub>C<sub>13</sub> 60 to 70 S RNA. At saturation, approx. 96 % of the <sup>3</sup>H-DNA probe hybridized to S<sub>2</sub>C<sub>13</sub> virus RNA (data not shown) showing that almost all of the <sup>3</sup>H-DNA is complementary to virus RNA. The digestion of S<sub>2</sub>C<sub>13</sub> <sup>3</sup>H-DNA product with S<sub>1</sub> nuclease showed it to be almost 98 % single-stranded. However, the proportion of the S<sub>2</sub>C<sub>13</sub> virus genome (60 to 70 S RNA) that is represented in the <sup>3</sup>H-DNA probe was not determined. No detectable hybridization of the <sup>3</sup>H-DNA probe was observed either with increasing amounts of (poly A) or with beef liver tRNA. Hybridization of the <sup>3</sup>H-DNA probe with (poly A) was carried out to test the probability that a portion of the <sup>3</sup>H-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.

Detection of virus specific RNA in control and regenerating livers

Virus-specific sequences in RNA extracted from control, sham-operated and regenerating BALB/c mouse livers were detected by hybridization to S<sub>2</sub>C<sub>13</sub> virus probe under conditions of RNA excess. The results presented in Fig. 2 show that while the RNAs extracted from control livers and livers of sham-operated mice hybridize at the saturating levels of 22 % of
Fig. 3. Melting curves of the hybrids produced by annealing of S₂Cl₃ virus RNA (●—●) and 20 h regenerating liver (BALB/c) RNA (○—○) with S₂Cl₃ virus ³H-DNA probe.

the ³H-DNA probe, RNAs from 12, 20 and 36 h regenerating livers saturate the probe at levels of 29 %, 37 % and 28 % respectively. The cytoplasmic RNA extracted from S₂Cl₃ cells yielded approx. a 69-5 % hybridization level (not shown in the figures). The precision of homology of the S₂Cl₃ virus DNA probe to both S₂Cl₃ virus RNA and regenerating liver RNA was assessed by determining the melting profiles of the DNA–RNA hybrids shown in Fig. 3. Tm values for the two hybrids, as well as the shapes of the melting curves, were similar which suggests that the hybrids formed between ³H-DNA probe and liver RNA are as well matched as the hybrids between DNA probe and S₂Cl₃ virus 60 to 70S RNA.

Although RNAs obtained from regenerating livers showed elevated hybridization values with virus DNA probe in comparison to control and sham-operated liver RNAs, the increase was most evident with the RNA from 20 h regenerating liver (Fig. 2). This shows that the extent of liver RNA–virus DNA hybridization attains its highest level just before the level of DNA synthesis in the regenerating liver reaches its maximum.

**DISCUSSION**

The data obtained from the present experiment reveal that although the endogenous S₂Cl₃ virus-specific RNA molecules are synthesized continuously in the mature liver of BALB/c mice, the RNA molecules synthesized in the regenerating liver hybridize the S₂Cl₃ ³H-DNA probe to a greater extent than the RNA from the mature liver. This increase in hybridization values is most evident with the RNA from the regenerating liver tissues
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collected at 20 h post-hepatectomy, just before the level of DNA synthesis in the regenerating liver reaches its maximum. Since almost all of the $^3$H-DNA probe used in this study is complementary to 60 to 70S RNA of the $S_2C_3$ virus the hybridizable RNA molecules in various liver samples are likely to be virus-specific. The homology of the $S_2C_3$ virus DNA probe to regenerating liver RNA, as evidenced by the melting profile analysis of the hybrid molecules, supports this conclusion. The increase in hybridization values with regenerating liver RNA may reflect an overall increase in the synthesis of RNA molecules already present in the mature liver in response to partial hepatectomy. On the other hand, due to the fact that virus $^3$H-DNA was hybridized with liver RNA in vast excess and saturation hybridization levels for RNA preparations from all liver samples were achieved (Fig. 2), the observed increase in hybridization values may reflect transcription of new virus sequences in the regenerating liver that are repressed in the normal liver. Church & McCarthy (1967a) have shown by using a competition hybridization technique, that re-activation of genes which are repressed in the adult liver takes place during liver regeneration. However, because of the lack of information on the uniformity of the virus $^3$H-DNA probe used in the present study and because reconstruction experiments utilizing normal and regenerating liver RNA were not carried out, it is not possible to distinguish between a quantitative and qualitative difference in the virus genome transcription pattern in normal and regenerating livers. In the presence of an asymmetric probe the increased level of hybridization may simply reflect the amplification of over-represented probe sequences. Nevertheless, the present study clearly indicates a change (either quantitative or qualitative) in the endogenous $S_2C_3$ virus genome transcription pattern in BALB/c liver following partial hepatectomy, which may have some functional significance.

Regenerating livers, like early embryos, undergo the process of regulated growth and development. The foetal enzymes which are present in early embryos have also been found in regenerating livers but not in adult livers (Abelev, 1971; Bonney et al. 1973). The competition hybridization studies, carried out by Church & McCarthy (1967b), have shown that embryonic mouse liver RNA is an efficient competitor for regenerating liver RNA, and it has been suggested that the characteristic regenerating liver RNA molecules may represent genes which are active during foetal liver development. The presence of high titres of group-specific antigen of the type-C RNA tumour virus in uninfected embryos of many strains of mice has been reported (Huebner et al. 1970). Our previous hybridization study (Mukherjee & Mobry, 1975) has shown an increased level of endogenous $S_2C_3$ virus-specific RNA synthesis in BALB/c embryos. These, along with the other observations made in the present study, support the view that endogenous type-C virus genomes may play a role in normal vertebrate development (Todaro & Huebner, 1972).

The study carried out by Hwang et al. (1974) shows that the changes in the template activity of chromatin and in the activities of enzymes necessary to support nuclear DNA synthesis in regenerating livers are sequential and related events. The RNA preparations from tissues having higher proliferative activity, such as spleen, small intestine, uterus and whole embryos of BALB/c mice, have been found to hybridize the $S_2C_3$ $^3$H-DNA probe to a greater extent than the RNA from tissues with low proliferative activity, such as kidney and liver (Mukherjee & Mobry, 1975). The results of the present experiment show that with the induction of rapid cell proliferation in the regenerating liver there is a concomitant increase in the synthesis of virus-specific RNA molecules in the hepatocytes. These observations may suggest the existence of a correlation between a change in the endogenous virus genome transcription pattern and cell proliferation.
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