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DNA Synthesis by RNA-Containing Tumor Viruses

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Abstract. Murine leukemia (Rauscher and Moloney strains) and sarcoma (Kirsten strain) virions, as well as the mammary tumor virus of mice, contain an RNA-dependent DNA polymerase. Optimal incorporation of deoxyribonucleoside triphosphates occurs at a critical detergent (Triton X-100) concentration (0.010–0.014%). At higher than optimal detergent concentrations the virion is seen to be disrupted and enzyme activity is lost. The virion, enzymatic activity, and newly synthesized DNA all cosediment in a sucrose gradient. Thus far the enzymatic activity has been found only in RNA viruses that have oncogenic properties.

The existence of an RNA-dependent DNA polymerase in RNA-containing tumor viruses has been postulated from indirect evidence.[1,2] More recently, Temin and Mizutani[3] and Baltimore[4] have reported the presence of such an enzymatic activity associated with Rous sarcoma virus and Rauscher leukemia virus preparations. The present studies were undertaken to investigate the generality of occurrence of this enzyme, and to study some of the characteristics of the reaction.

Materials and Methods. Viruses: The Kirsten strain of murine sarcoma virus (Ki-MSV)[5] was grown in NIH/3T3 cells.[6] Culture fluids were harvested 2 days after a medium change and centrifuged at 10,000 × *g* for 15 min to remove cell debris. The supernatant was then centrifuged through 10% sucrose for 2 hr at 30,000 × *g* in a Spinco no. 30 rotor. The pelleted viruses were resuspended in 0.01 M Tris·HCl, pH 7.4–0.10 M NaCl–6 mM β-mercaptoethanol, to a final volume of $^1/_{50}$th of the initial volume, and stored at −70°C. Rauscher leukemia virus (R-MuLV), obtained as a band on density gradient centrifugation of the plasma of leukemia-infected mice, was supplied by the Resources and Logistics Segment of the National Cancer Institute. It had a titer of $10^{5.5}$ spleen-enlarging units/ml. A variant of Rauscher leukemia virus, adapted to grow in human cells, was concentrated from supernatant fluids of human embryonic kidney cells.[7] Moloney leukemia virus (M-MuLV), grown in the JLSV9 BALB/c mouse line[8], and Rauscher leukemia virus, grown in BALB/c spleen-thymus line JLSV5,[9] were supplied by Electro-Nucleonics Laboratories (Bethesda, Md.); these viruses had been purified twice on density gradients. The Gardner-Arnstein strain of feline sarcoma virus (G-FeSV), isolated from a spontaneous sarcoma,[10] was also prepared and purified on a gradient by Electro-Nucleonics. The murine mammary tumor virus (MTV) was obtained after isopycnic banding in a density gradient[11] through the courtesy of Mel-Labs, Inc., Springfield, Va. Sendai virus was grown in eggs as described previously.[12] The virus was harvested in the allantoic fluid of embryonated chick eggs and concentrated 100 times by centrifugation at 25,000 × *g* for 1 hr (Spinco no. 30 rotor). Respiratory syncytial virus and influenza virus (density-gradient purified) were supplied by Dr. B. Gerwin (Molecular Anatomy Program, Union Carbide, Rockville, Md.). Mouse lymphocytic

choriomeningitis virus, with a titer of more than 10^8 plaque forming units/ml, was supplied by Dr. W. Rowe (NIH). Newcastle disease virus was supplied by Dr. K. Blackman (Mel-Labs, Inc., Springfield, Va.).

To prepare radioactively-labeled Ki-MSV, [^{14}C]uridine (sp act 50 mCi/mmol, New England Nuclear Corp., Boston, Mass.) was added at a concentration of 10.0 μCi/ml to logarithmically growing NIH/3T3 cells chronically infected with Ki-MSV. Culture fluids were harvested at 24 hr and centrifuged to remove debris, then further purified by sedimentation through 10% sucrose at 30,000 $\times$ g for 2 hr (Spinco no. 30 rotor). The virus, concentrated fifty times in 0.01 M Tris·HCl, pH 7.4–0.10 M NaCl–6 mM β-mercaptoethanol, was stored at −70°C.

XC plaque assay: The XC plaque test of Rowe *et al.* (to be published) was used for murine leukemia viruses (MuLV). The test is based on the observation of Klement *et al.*[13] that the rat tumor cell, XC[14], undergoes syncytium formation in the presence of MuLV-producing cells. This method gives titers for the MuLV stocks used in these studies that are comparable to those obtained by the standard complement fixation assay.[15] For the assay, logarithmically growing BALB/3T3 and NIH/3T3 cells[6] were infected with 0.5 ml of virus after treatment of the cells with diethylaminoethyl-dextran.[16] Infected cultures were overlaid with 1 $\times$ 10^6 XC cells after 7 days and were fixed in methanol and stained with Harris hemotoxylin 3 or 4 days later. Plaques were scored with the aid of a dissecting microscope.

DNA polymerase assay: RNA-dependent DNA polymerase activity was assayed by a modification of the method described by Baltimore[4] [pyrophosphate was omitted from the trichloroacetic acid (TCA) precipitation step]. Details are described in the appropriate legends. Dithiothreitol was purchased from Calbiochem, Los Angeles, Calif. Nonradioactive deoxyribonucleoside triphosphates were obtained from P-L Biochemicals, Milwaukee, Wis. [^{3}H]Deoxyadenosine-5′-triphosphate (sp act 13.1 Ci/mmol) and [^{3}H-methyl]thymidine triphosphate, [^{3}H]dTTP (sp act 11.1 Ci/mmol) were from New England Nuclear, Boston, Mass.

Results. Kinetics of incorporation: A stock of Ki-MSV grown in tissue culture was used to determine the time course of [^{3}H]dATP incorporation into cold TCA precipitable counts. As shown in Fig. 1, the reaction, including all

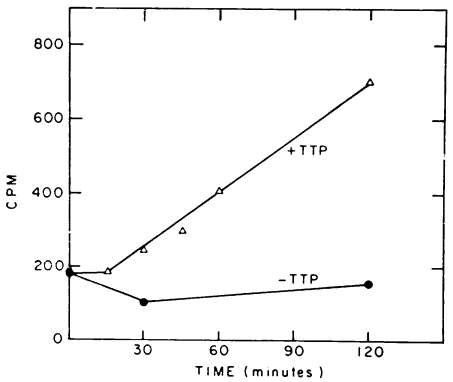

FIG. 1. Kinetics of DNA synthesis. Each reaction mixture was incubated at 37°C for the times indicated and contained in 0.05 ml: 0.05 M Tris·HCl, pH 8.3; 6 mM magnesium acetate; 0.06 M NaCl; 0.02 M dithiothreitol; 0.01% (v/v) Triton X-100; 80 mM each of three nonradioactive deoxyribonucleoside triphosphates; 35 μM thymidine triphosphate, at a specific activity of 1.2 Ci/mol; and Kirsten sarcoma virus (5 $\times$ 10^4 focus-forming units).

four deoxyribonucleoside triphosphates, Ki-MSV, and [^{3}H]dATP, in the presence of 0.01% Triton X-100, showed a delay of 15–30 min before incorporation increased linearly. The incorporation of radioactivity into TCA-precipitable counts then continued to increase linearly for at least 2 hr. The reason for the lag is not clear but may be either permeability changes in the outer membrane of the virion, or some specific event in the initiation of transcription as in the

case of the vaccinia core polymerase.[17] In the absence of one of the four essential deoxyribonucleoside triphosphates (dTTP), there was no detectable incorporation above the level at zero time.

The *in vitro* DNA polymerase activity was temperature-dependent. Table 1 shows that optimal activity was obtained at about 37°C, with less incorporation

TABLE 1. *Effect of temperature on DNA synthesis by Moloney leukemia virus.**

Temperature (°C)	Cpm incorporated†
24	213
32	822
37	2750
41	2570

* Incubated for 90 min at the temperatures indicated, 0.05 ml, components noted in Fig. 2*A*.
† Zero time value of 202 cpm was subtracted.

at lower temperatures. Very little activity was detectable after incubation for 2 hr at room temperature (23°C). In other experiments, a broad optimum concentration for sodium chloride was found between 0.06 and 0.14 M, and for magnesium acetate, the optimum concentration was 0.006–0.010 M. In agreement with Baltimore[4] no incorporation was detectable with ribonucleoside triphosphates or with the deoxyribonucleoside triphosphates after RNase A treatment of the virion.

Dependence of the reaction on Triton X-100 concentration: In contrast to the results of a previous report,[4] the viruses we tested showed little or no activity in the absence of Triton X-100 in the reaction mixture. As shown in Fig. 2, activity showed a very sharp dependence on Triton X-100 concentration. Optimal activity was found in a range from 0.010 to 0.014% for both the R-MuLV and M-MuLV preparations. A slight increase above the optimal concentration of the detergent resulted in markedly lower activity. These findings make necessary careful determination of the optimal Triton concentration for each virus to be tested. The stimulation of activity at optimal Triton levels was as much as 200 times the control values obtained in the absence of one of the 4 deoxynucleotide triphosphates (either dATP or dTTP).

Cosedimentation of virion, enzymatic activity, and the DNA product: In Fig. 3, the Triton-treated Rauscher leukemia virion and the DNA product of the reaction were analyzed on sucrose gradients. Virions were treated for 60 min with Triton X-100 and then sedimented in a sucrose gradient. In Fig. 3*A*, DNA polymerase activity was found in a sharp peak at a density of 1.16 g/cm^3, cosedimenting with ^{14}C-labeled virus that had been included in the reaction mixture. The results suggest that under these conditions Triton X-100 does not solubilize the DNA polymerase from the virus. In addition, since the detergent and the virus are presumably separated on the gradient, Triton appears not to be required for DNA polymerase activity but rather to make the substrates accessible to the enzyme. In Fig. 3*B*, the DNA product of the reaction can be found also to cosediment with the marker virion and with the DNA polymerase activity seen in Fig. 3*A*. The results indicate that virion, enzymatic activity, and the DNA product synthesized all cosediment in a sucrose gradient, and sug-

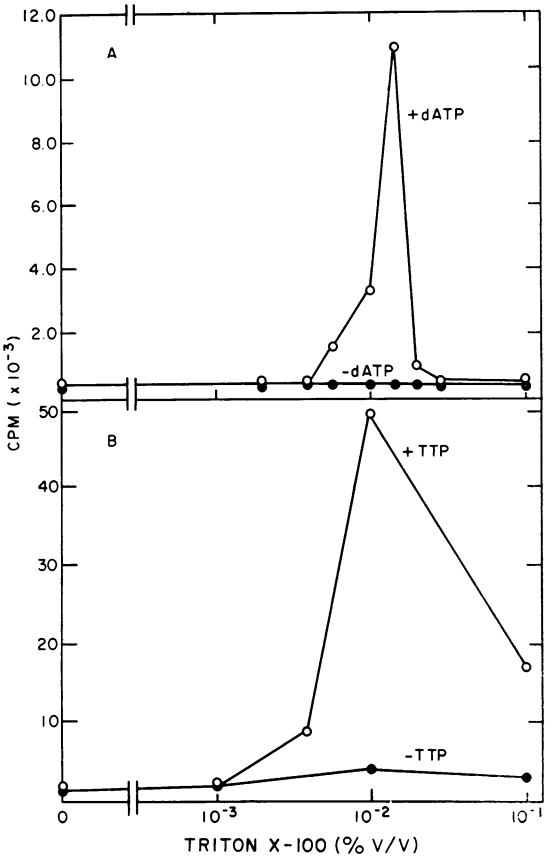

FIG. 2. Effect of Triton X-100 on DNA synthesis. (*A*) Each reaction mixture was incubated for 90 min at 37°C and contained in 0.05 ml: 0.05 M Tris·HCl, pH 8.3; 8 mM magnesium acetate; 0.08 M NaCl; 0.02 M dithiothreitol; 80 mM each of three nonradioactive deoxyribonucleoside triphosphates; 10 μM dATP, specific activity 13.1 Ci/mmol. The source of enzyme was the Moloney strain of murine leukemia virus (1.5 × 10^6 plaque-forming units).

(*B*) Reaction mixtures as in (*A*) except that 10 μM thymidine triphosphate (sp act, 11.1 Ci/mmol) was substituted for dATP. The source of enzyme was Rauscher leukemia virus (7.0 × 10^5 plaque-forming units).

gest that the three are part of a ternary complex. Whether the newly-formed DNA is synthesized from the entire RNA genome or only part of it is presently being studied.

DNA polymerase activity in RNA-containing virions: A large number of RNA-containing viruses, including several non-oncogenic RNA viruses, were tested for activity in the DNA polymerase assay. In preliminary experiments, the rate of incorporation was found to be linearly proportional to the concentration of virus. Table 1 shows that the rate of incorporation varied greatly, depending on the particular virus preparation used. The most active was Rauscher leukemia virus, obtained from the plasma of leukemic mice. Over 64,000 cpm was incorporated as compared to 430 cpm for the control. Other preparations showing high activity included a Moloney leukemia virus grown in mouse cells, a Kirsten sarcoma virus grown in rat cells, and a Rauscher leukemia virus grown in human cells. Activity was also observed with a preparation of G-FeSV (Gardner-Arnstein strain). It is not clear, as yet, whether the differences in activities among the viruses reflect differences in the manner of their preparation or whether they reflect intrinsic differences in the viruses themselves.

Of great interest was the presence of activity in MTV. While the growth of

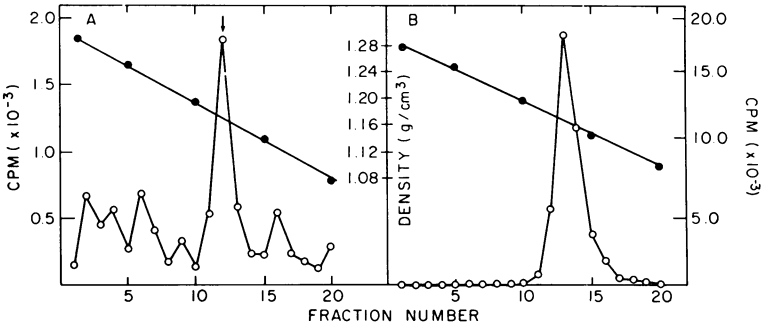

FIG. 3. Sucrose gradient analysis of Triton-treated Rauscher Leukemia Virus. (*A*) Enzyme assay of gradient fractions: Reaction mixture incubated 60 min at 37°C, 0.30 ml, as noted in Fig. 2*A* with 0.01% (v/v) Triton X-100, 2×10^6 plaque-forming units of Rauscher leukemia virus, and 2000 cpm of [^{14}C]uridine-labeled Kirsten leukemia virus as marker. dTTP was omitted from the reaction mixture. After incubation, the reaction mixture was placed on a 15–60% sucrose gradient containing 0.08 M NaCl; 8 mM magnesium acetate; 0.01 M Tris ·HCl, pH 8.3; and 6 mM β-mercaptoethanol, and centrifuged at full speed in a Spinco SW 41 rotor at 4°C for 150 min. Fractions of 0.5 ml were collected from the bottom of the tube, and 25 μl of each fraction was assayed for polymerase activity. The zero time value of 200 cpm is subtracted from all values.

(*B*) Gradient analysis of newly synthesized DNA: Reaction mixture as in 3*A* except [methyl-^{3}H]dTTP was also included. After incubation, the reaction was layered on a sucrose gradient, sedimented, and collected as above. Each fraction was analyzed for DNA by precipitation with cold TCA and collection of the precipitate on cellulose nitrate filters.

leukemia and sarcoma viruses is known from cell culture studies to require DNA synthesis subsequent to infection,[18,19] comparable studies have not been performed with MTV because of the absence of satisfactory tissue culture assays for this virus. Thus, the activity in MTV suggests that this RNA tumor virus, too, contains an RNA-dependent DNA polymerase. It was necessary to rule out the presence of contaminating leukemia virus in the MTV preparation, since the C_3H mouse strain is known to carry C-type RNA viruses. Infectious MuLV in the MTV stock was assayed by the XC plaque test, complement fixation, and a fluorescent antibody method (Rowe *et al.*, to be published); all were negative with the preparation shown in Table 2. Electron microscopy of negatively-stained MTV virus pellets revealed the presence only of spiked virus particles characteristic of MTV.[20]

Non-oncogenic RNA viruses that replicate by budding from cell membranes were similarly tested. A preparation of Sendai virus, which contained at least ten times the number of physical particles as compared to any of the other viruses, showed no incorporation above background at several concentrations of detergent. Similarly, respiratory syncytial virus, Newcastle disease virus, lymphocytic choriomeningitis virus, and influenza virus were inactive.

Discussion. The finding of an RNA-dependent DNA polymerase by Temin and Mizutani[3] and by Baltimore[4] is supported and extended by this and other papers submitted simultaneously.[21,22] That such template activity is found with a variety of oncogenic RNA viruses, produced both in the animal and in cell culture, but is not found with several non-oncogenic RNA viruses (those reported here, and vesicular stomatitis virus[4]) increases the likelihood that the

TABLE 2. *DNA synthesis by RNA virions.**

RNA Virus	Particles/ml†	Infectivity (XC plaques/ml)	Cpm incorporated −dATP	Cpm incorporated +dATP
Oncogenic				
C-type leukemia				
Rauscher murine (RMuLV)	...	$10^{8.7}$	430	64,000
Moloney murine (MMuLV)	5×10^{11}	$10^{7.5}$	401	5,900
Rauscher murine (human adapted)	...	...	295	2,000
C-type sarcoma				
Kirsten murine (Ki-MSV)	1×10^{9}	$10^{7.3}$	350	1,050
Gardner feline (G-FeSV)	1×10^{9}	...	250	548
B-type				
Mammary tumor (MTV)	1×10^{11}	0	301	1,050
Non-oncogenic				
Sendai virus	5×10^{12}	...	205	198
Respiratory syncytial virus	$>10^{9}$	...	173	161
Lymphocytic choriomeningitis virus	$>10^{11}$	...	195	187
Influenza virus	$>10^{11}$	...	188	192
Newcastle disease virus	$>10^{11}$	...	202	196

* Incubated for 120 min, 0.05 ml, components noted in Fig. 2A.
† Estimated by electron microscopy.

activity is related to the oncogenicity of the viruses. However, since the originally described *DNA*-dependent DNA polymerase of *E. coli* is now thought not to constitute part of the cellular machinery for replication of the *Escherichia coli* genetic material *in vivo*[23], one must be cautious in assigning a physiological role to this RNA-dependent polymerase.

For the murine viruses, M-MuLV, R-MuLV, and Ki-MSV, the range of optimal Triton X-100 concentration was 0.010–0.014%. This very narrow range of detergent concentration necessary to demonstrate activity makes negative experiments difficult to interpret. Electron microscopy shows that below the optimal detergent concentration, the viral envelope remains intact and impermeable to uranyl acetate or to phosphotungstic acid (unpublished results). At the detergent concentration that gives maximal activity, the particles are sufficiently damaged so that the stain penetrates, but the cores remain intact. Further increases in detergent concentration are associated with a loss of enzyme activity, and disruption of the particle can be seen. Thus it appears that under these conditions an intact particle is needed for DNA synthesis but that the outer envelope must be altered in its permeability properties to demonstrate the activity *in vitro*. This, coupled with the inability to separate the enzyme from a virion of buoyant density of 1.16 g/cm^3 after detergent treatment, provides further evidence that the enzyme activity is located within the virion and is not adventitiously adsorbed to the surface of the particle.

The mouse mammary tumor virus, a B-type particle,[24] also showed RNA-dependent DNA polymerase activity. This virus, like the C-type RNA viruses, contains high molecular weight (>60S) single-stranded RNA.[25] There is as yet no tissue culture assay for MTV infectivity; enzyme activity then must be expressed in terms of physical particles. Three different MTV preparations contained polymerase activity. Each was tested for leukemia virus by immunodiffusion and complement fixation, and by biological assays (XC plaque test

and fluorescent focus assay). One of the preparations that showed a high level of DNA polymerase activity (>20,000 cpm above the control) contained leukemia virus. With this preparation, therefore, it was not possible to conclude that all of the activity was due to MTV. The other two preparations showed no detectable MuLV by the tests described and showed an abundance of characteristic B-type particles of MTV by electron microscopy. This leads us to conclude that MTV itself contains the enzymatic activity for RNA-dependent DNA synthesis.

Recently, an RNA-containing virus that is structurally very similar to MTV has been found in two carcinoma cell cultures from human breast[26,27] and has also been observed in the milk of certain women with breast cancer.[28] Should the production of a DNA intermediate be a property restricted to tumor viruses, the presence of such activity in viruses of human origin would increase the likelihood that such viruses play an etiologic role in human cancer.

Note Added in Proof. The enzyme activity has been separated from the intact virion and is found in the nucleoid.[29] Manganese rather than magnesium is the preferred divalent cation for DNA synthesis, especially with synthetic RNA templates.

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Abbreviations: TCA, trichloroacetic acid; virus strains as defined in Table 2; MuLV, murine leukemia virus.

[1] Temin, H. M., *Nat. Cancer Inst. Monogr.*, **17**, 557 (1964).
[2] Bader, J. P., *Virology*, **29**, 444 (1966).
[3] Temin, H. M., and S. Mizutani, *Nature*, **226**, 1211 (1970).
[4] Baltimore, D., *Nature*, **226**, 1209 (1970).
[5] Kirsten, W. H., and L. A. Mayer, *J. Nat. Cancer Inst.*, **39**, 311 (1967).
[6] Jainchill, J. L., S. A. Aaronson, and G. J. Todaro, *J. Virol.*, **4**, 549 (1969).
[7] Wright, B. S., and W. Korol, *Cancer Res.*, **29**, 1886 (1969).
[8] Wright, B. S., and J. C. Lasfargues, *Nat. Cancer Inst. Monogr.*, **22**, 685 (1966).
[9] Wright, B. S., P. A. O'Brien, G. P. Shibly, S. A. Mayyasi, and J. C. Lasfargues, *Cancer Res.*, **27**, 1672 (1967).
[10] Gardner, M. B., R. W. Rongey, P. Arnstein, J. D. Estes, P. S. Sarma, R. J. Huebner, and C. G. Rickard, *Nature*, **226**, 807 (1970).
[11] Bond, H. E., and W. T. Hall, *J. Nat. Cancer Inst.*, **43**, 1073 (1969).
[12] Takemoto, K. K., G. J. Todaro, and K. Habel, *Virology*, **35**, 1 (1968).
[13] Klement, V., W. P. Rowe, J. W. Hartley, and W. E. Pugh, *Proc. Nat. Acad. Sci. USA*, **63**, 753 (1969).
[14] Svoboda, J., P. P. Chyle, D. Simkovic, and I. Hilgert, *Folia Biol.*, **9**, 77 (1963).
[15] Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner, *Proc. Nat. Acad. Sci. USA*, **53**, 931 (1965).
[16] Vogt, P. K., *Virology*, **33**, 175 (1967).
[17] Kates, J., and J. Beeson, *J. Mol. Biol.*, **50**, 1 (1970).
[18] Temin, H. M., in *Biology of Large RNA Viruses*, ed. R. Barry and B. McKay (New York Academic Press, 1970), in press.
[19] Bader, J. P., in *The Biochemistry of Viruses*, ed. H. B. Levy (New York: Marcel Dekker, 1969), p. 293.
[20] Moore, D. H., N. H. Sarkar, and J. Charney, *J. Nat. Cancer Inst.*, **44**, 965 (1970).
[21] Hatanaka, M., R. J. Huebner, and R. V. Gilden, *Proc. Nat. Acad. Sci. USA*, (1970) in press.
[22] Green, M., M. Rokatunda, and K. Fujinaga, *Proc. Nat. Acad. Sci. USA* (1970) in press.
[23] deLucia, P., and J. Cairns, *Nature*, **224**, 1164 (1969).
[24] Bernard, W., *Cancer Res.*, **20**, 712 (1960).

[25] Robinson, W. S., H. L. Robinson, and P. H. Duesberg, *Proc. Nat. Acad. Sci. USA*, **58** 825 (1967).
[26] Todaro, G. J., V. Zeve, and S. A. Aaronson, *Nature*, **226**, 1047 (1970).
[27] Todaro, G. J., V. Zeve, and S. A. Aaronson, *In Vitro*, in press.
[28] Moore, D. H., N. H. Sarkar, C. E. Kelly, N. Pillsbury, and J. Charney, *Tex. Rep. Biol. Med.*, **27**, 1027 (1969).
[29] Gerwin, B., G. J. Todaro, V. Zeve, E. Scolnick, and S. A. Aaronson, *Nature*, in press.