stratospheric H_2O mass mixing ratio [$c_0(H_2O)$] (Fig. 2). This peak is a consequence of the decreased efficiency of the cold trap at high H_2O levels (*8, 12*). At the high point of this curve the oceans could be depleted of water in about 9 billion years if the diffusion-limited hydrogen escape flux was achieved. A 30° increase in T_s, which could result from a mere 10% increase in solar flux according to our model, would cause $c_0(H_2O)$ to rise to about 0.1 at this CO_2 pressure. The oceans would then disappear in only a few hundred million years, as may have happened on Venus (*8*). The present atmosphere might therefore be said to be marginally stable against water loss at CO_2 pressures of 0.1 to 1 bar. At still higher CO_2 pressures, the stratosphere should once again be dry and the oceans should be more stable.

Our calculations do not prove that the early atmosphere must have been hot. The question of how CO_2 was originally partitioned between the atmosphere, ocean, and solid planet is complex, and it is possible that the early atmosphere was much less massive than we have assumed. We have shown, however, that a dense CO_2 atmosphere could have existed on the early earth without violating any known constraints on the planet's subsequent evolution.

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11. Rayleigh scattering by CO_2 is particularly important because its scattering cross section is about 2.5 times as high as that of air [(H. C. Van de Hulst, in *The Atmospheres of the Earth and Planets*, G. P. Kuiper, Ed. (Univ. of Chicago Press, Chicago, 1957), p. 49]. Our cross sections are the same as those used in another recent model of the climatic effects of large CO_2 increases [I. M. Vardavas and J. H. Carver, *Planet. Space Sci.* **32**, 1307 (1984)].
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$$r = r_0\left[\frac{p/p_s - 0.02}{1 - 0.02}\right]^{\Omega} \quad (1)$$

where p is pressure, p_s is surface pressure, and r_0 (= 0.8) is the surface relative humidity. The parameter Ω allows the relative humidity of the upper troposphere to vary. When $\Omega = 1$, Eq. 1 reduces to the empirical formula of Manabe and Wetherald [*J. Atmos. Sci.* **24**, 241 (1967)], which is used in many one-dimensional climate models. When $\Omega = 0$, the troposphere is 80% saturated throughout. This formulation follows a suggestion by R. D. Cess [*ibid.* **33**, 1831 (1976)] which he made on the basis of observed latitudinal gradients in T_s and r. He proposed that $\Omega = 1 - 0.03(T_s - 288\ K)$. We have replaced this formula with

$$\Omega = 1 - \frac{f_{sat}(H_2O) - f_p}{0.1 - f_p}$$

with $0 \leq \Omega \leq 1$ and where $f_{sat}(H_2O)$ [$= p_{sat}(H_2O)/p_s$] is the saturation H_2O mixing ratio at the surface and f_p (= 0.0166) is the value of $f_{sat}(H_2O)$ for the present atmosphere (T_s = 288 K). Ω is assumed to be unity for $f_{sat}(H_2O) < f_p$ and 0 for $f_{sat}(H_2O) > 0.1$. We used this equation instead of Cess's formula because we feel that the behavior of water vapor in an atmosphere is more closely related to its mixing ratio than to surface temperature (*12*). It gives approximately the same result as Cess's formula for a 1-bar atmosphere, but yields a slower increase in r at higher surface pressures.
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Ultraviolet Irradiation Transforms C3H10T1/2 Cells to a Unique, Suppressible Phenotype

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Transformation of C3H10T1/2 cells by exposure to ultraviolet (UV) irradiation followed by tetradecanoyl phorbol acetate (TPA) has been used as a model of two-stage carcinogenesis. However, cells cloned from UV-TPA–induced foci (UV-TDTx cells) had a unique phenotype. Cloned UV-TDTx cells appeared transformed in pure culture but were unable to form foci when cocultured with C3H10T1/2 cells. However, in the presence of TPA, UV-TDTx cells form foci in mixed culture with C3H10T1/2 cells. This phenotype was the only one observed for UV-TPA transformants. These data suggest that (i) communal suppression of cell division is a discrete phenomenon that must be overcome as one step in the multistage process of transformation, and (ii) this protocol permits the routine isolation of transformed cells responsive to density-dependent growth suppression.

C3H10T1/2 CELLS (*1*) ARE A MAJOR cell culture model for the study of transformation by chemical carcinogens (*2, 3*) and ionizing radiation (*4, 5*). The presence of dominant transforming oncogenes from high-dose methylcholanthrene (MCA) C3H10T1/2 transformants has been demonstrated (*6*) and the genes identified as altered K-*ras* (*7*). A two-step C3H10T1/2 transformation protocol was developed that reflects in vivo initiation and promotion (*8*). After low doses of carcinogens or radiation, C3H10T1/2 cells require subsequent exposure to a tumor promoter for transformation. Although numerous studies have utilized C3H10T1/2 cells to define and characterize initiators and promoters, the molecular changes required for two-stage transformation are unknown. We planned to isolate two-stage C3H10T1/2 transformants and search by DNA-mediated transfection (*6, 7*) for dominant transforming oncogenes. However, two-stage C3H10T1/2 transformants had properties not shared with most single-step transformants isolated in response to chemical carcinogens or ionizing irradiation (*2–5*).

Plates with 2000 C3H10T1/2 cells were

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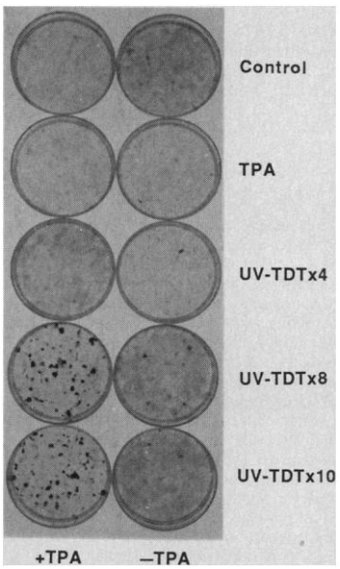

Fig. 1. Secondary focus-forming assay of uncloned UV-TDTx cells. From each of ten uncloned UV-TDTx cultures 200 cells were plated with 1800 C3H10T1/2 cells and cultured either in the presence or in the absence of TPA (100 ng/ml). Similar cocultures were performed with untreated C3H10T1/2 cells and cells previously grown in the presence of TPA or exposed to UV irradiation. After 3 weeks the cells were stained.

exposed to ultraviolet (UV) irradiation alone, tetradecanoyl phorbol acetate (TPA) alone, or UV irradiation followed by TPA (*9*). Foci of dense, rounded cells were rarely observed on untreated plates, or on UV- or TPA-treated plates. Plates exposed first to UV irradiation and then to TPA developed foci after 4 to 6 weeks. Single foci and surrounding cells from each of ten plates were isolated with cloning cylinders and subcultured. We refer to these as UV-TDTx (TPA-dependent transformed) cells. When these ten cultures grew to saturation density, no focus formation occurred; the mixed cell populations were indistinguishable from C3H10T1/2 cultures. We then postulated that focus formation by UV-TDTx cells might be dependent on the continued presence of TPA. "Secondary focus-forming assays"—mixed cultures with 1800 C3H10T1/2 and 200 uncloned UV-TDTx cells—were plated in 60-mm dishes. Two plates were cultured with TPA and two plates were cultured without tumor promoter. No foci occurred, in the presence or absence of TPA, for seven of the C3H10T1/2 + UV-TDTx mixed cultures (see UV-TDTx4 in Fig. 1). In contrast, for three UV-TDTx cultures "secondary" foci were present after 3 to 4 weeks in the presence of TPA, but not in its absence (see UV-TDTx8, UV-TDTx10 in Fig. 1). Three uncloned cultures from UV + TPA two-stage foci appeared, therefore, to contain "initiated" cells, that is, cells able to form foci only when exposed to TPA (*10*).

Clonal lines established by limiting dilution from cultures of UV-TDTx1, UV-TDTx8, and UV-TDTx10 demonstrated the TPA-dependent focus-forming phenotype in secondary focus-forming assays (Fig. 2). In contrast, focus formation by MCA-Tx1 (a high-dose MCA transformant) was unaffected by TPA. "Secondary" focus formation was proportional to the number of cloned UV-TDTx cells present when the total number of cells plated was 2000 and the UV-TDTx cell number was varied between 5 and 500. At this point we concluded that we had established clonal, stable "UV-initiated" cell lines that require TPA for expression of the transformed phenotype.

Because we regarded UV-TDTx clones as "initiated" but not transformed, they were routinely passaged at low cell density to prevent selection for transformation after growth arrest. In contrast to the uncloned UV-TDTx populations, pure cultures of cloned UV-TDTx cells appeared transformed when allowed to grow to confluence. Mixed UV-TDTx + C3H10T1/2 cultures were prepared at higher ratios than those used in the experiment shown in Fig. 2. UV-TDTx10e cells in pure culture appear transformed in the presence or absence of TPA (Fig. 3). Even at a ratio of UV-TDTx10e to C3H10T1/2 of 3:1 the C3H10T1/2 cells suppressed, to some degree, expression of transformation by UV-TDTx10e cells. Similar results occurred with UV-TDTx1 and UV-TDTx8 clones. We conclude that (i) UV-TDTx clones appear transformed when grown in pure culture; (ii) in mixed culture C3H10T1/2 cells can suppress focus formation by UV-TDTx cells, and (iii) TPA can block the C3H10T1/2 suppression of focus formation by UV-TDTx cells.

To determine whether all foci produced by the two-stage UV-TPA protocol can be suppressed by C3H10T1/2 cells, 20 plates of C3H10T1/2 cells were carried through the UV-TPA protocol (Fig. 4). Other plates received no treatment, UV irradiation alone, or TPA alone. After 7 weeks, all cells from each dish were removed with trypsin and, in each case, four new plates were each seeded with 10,000 cells. Two plates were then cultured without TPA; two were cultured with TPA. Thus, cells from each focus on the original dishes were examined for subsequent suppression of focus formation by C3H10T1/2 cells in the absence of TPA. Replated cultures from untreated, UV-treated, or TPA-treated plates had no foci, either in the presence or absence of TPA. In contrast, foci occurred in the presence of TPA in replated cultures from 14 of the 20 dishes exposed first to UV irradiation and then to TPA. In no case, however, were foci present in the absence of TPA. In this experiment we examined a minimum of 14 independent transformations as a result of the two-stage UV-TPA protocol. (Many original UV + TPA–treated plates had multiple foci.) We conclude that UV-TPA two-stage transformation of C3H10T1/2 cells rarely, if ever, induces foci whose transformed phenotype cannot be suppressed by C3H10T1/2 cells in subsequent subculture.

Suppression of focus formation by "normal" cells has been observed in other cell culture systems (*11*). However, the UV-TDTx transformants differ from previously

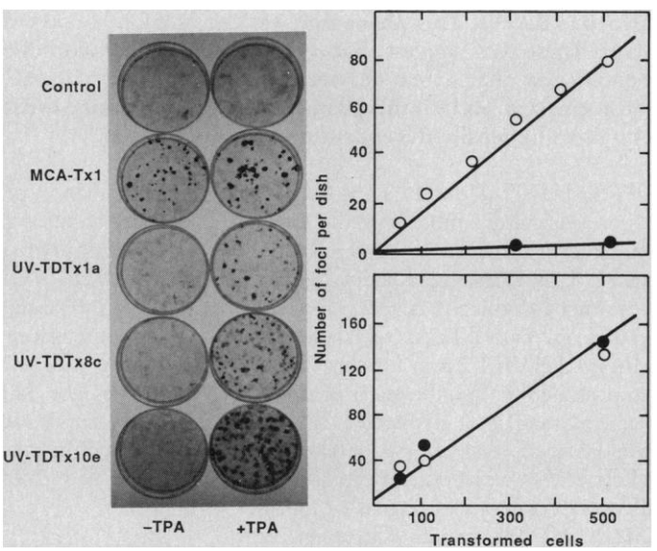

Fig. 2. (Left panel) Secondary focus-forming assay of UV-TDTx clones. Two hundred clonal UV-TDTx cells and 1800 C3H10T1/2 cells were cocultured either in the presence or in the absence of TPA (100 ng/ml). A secondary focus-forming assay with MCA-Tx1, a single-step MCA transformant, is also shown. "Control" cells are C3H10T1/2 cells. (Right panel) Focus formation is proportional to the number of UV-TDTx cells. Duplicate dishes were plated with a mixed population of either UV-TDTx10e (top) or MCA-Tx1 (bottom) cells and C3H10T1/2 cells totaling 2000 cells, and cultured either in the presence (open circles) or in the absence (closed circles) of 100 ng TPA per milliliter. Plating efficiencies for C3H10T1/2 and the UV-TDTx cells are approximately 20%. Similar results were obtained with UV-TDTx1 and UV-TDTx8 clones.

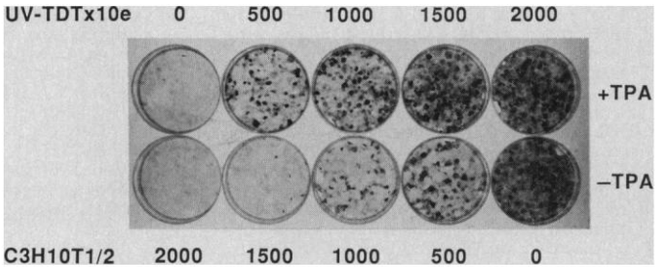

Fig. 3. Clonal UV-TDTx cells in pure culture exhibit a transformed phenotype. UV-TDTx10e and C3H10T1/2 cells were plated in the numbers shown, grown in the presence or absence of TPA (100 ng/ml) for 4 weeks, then stained.

characterized transformants; inhibition of UV-TDTx focus formation is complete at ratios of normal to transformed cells that gave only partial suppression in other systems, and the TPA concentration necessary to overcome suppression is substantially lower for UV-TDTx cells. Inhibition by C3H10T1/2 cells of focus formation has also been observed for selected MCA C3H10T1/2 transformants (*12*). The interactions of UV-TDTx cells with C3H10T1/2 cells differed from that observed with single-step MCA-transformed C3H10T1/2 cells: (i) the majority of MCA-induced C3H10T1/2 transformants formed foci in mixed culture, whereas every UV-TDTx transformant was suppressed by C3H10T1/2 cells; (ii) growth conditions exist (for example, culture in 5% serum) where C3H10T1/2 cells suppress focus formation by UV-TDTx cells, but have little effect even on these selected MCA transformants (*12, 13*); and (iii) TPA elicited focus formation in mixed UV-TDTx/C3H10T1/2 cultures, but did not strongly influence focus formation by MCA C3H10T1/2 transformants in mixed culture. Mehta *et al.* (*13*) report that in the case of the selected MCA transformants, suppression of the transformed phenotype is mediated by junctional communication, a phenomenon normally blocked by TPA (*14*).

Two-stage transformation of C3H10T1/2 cells has been used to identify initiators, identify promoters, and describe temporal and concentration-dependent relationships between these agents. Two-stage transformation assays are usually stained and scored; cells from two-stage foci have not been further characterized. It has been assumed that two-stage transformants would be similar to single-step transformants. Our data lead us to different conclusions. The two-stage UV-TPA protocol routinely results in C3H10T1/2 cells that exhibit a transformed phenotype in pure culture but are susceptible to suppression of the focus-forming phenotype by C3H10T1/2 cells. Heidelberger and his colleagues (*9*) suggested that UV irradiation is a "pure" initiator, unable to transform C3H10T1/2 cells. We suggest that UV irradiation can transform C3H10T1/2 cells to a unique phenotype. However, UV-induced transformants are not observed in an irradiated culture as foci; expression of the UV-transformed phenotype is suppressed by surrounding C3H10T1/2 cells. Only when TPA relieves the suppressive effect of C3H10T1/2 cells is focus formation observed. This phenotype is by far the most frequent, if not the only one, observed in response to UV irradiation followed by TPA. We postulate that suppression of cell division by interactions among adjacent cells is a discrete phenomenon that must be overcome as one of the steps in multistage carcinogenesis. The UV-TPA protocol allows us to routinely trap altered C3H10T1/2 cells at a point in the transformation pathway where suppression is still effective; either an exogenous agent such as TPA or a subsequent cellular event is required to abrogate the suppression mechanism and permit UV-TDTx cells to form foci in the presence of normal cells.

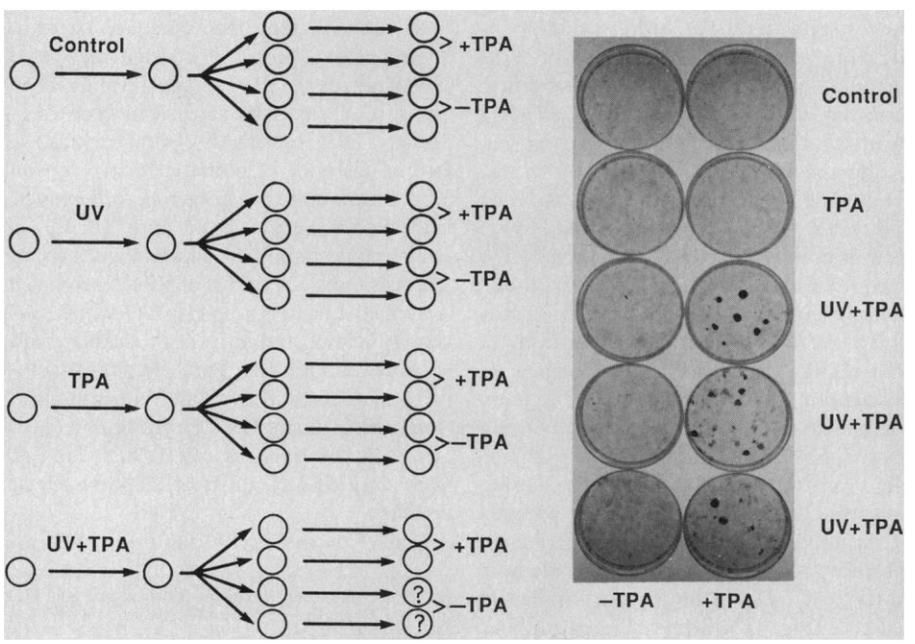

Fig. 4. Replating experiment to determine if focus formation by all UV-TDTx transformants can be suppressed in mixed culture. (Left panel) Protocol for the experiment. Cells received no treatment, UV irradiation alone, TPA alone, or UV + TPA. After 7 weeks, cells were removed with trypsin and, for every culture, four new plates were each seeded with 10,000 cells. Plates were cultured in either the presence or the absence of TPA. (Right panel) Representative replated cultures from different dishes.

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9. UV radiation was selected as the initiator because Heidelberger and his colleagues [C. Heidelberger, S. Mondal, A. R. Peterson, in *Carcinogenesis: Mechanisms of Tumor Promotion and Cocarcinogenesis*, T. J. Slaga, A. Sivak, R. K. Boutwell, Eds. (Raven, New York, 1978), vol. 2, p. 197] reported that UV irradiation alone at any dose did not induce foci. We used 100 ergs per square millimeter of 254-nm UV irradiation as initiator and 100 ng TPA per milliliter as promoter. Culture medium and fetal calf serum were from Gibco, Grand Island, NY; TPA was from Chemicals for Cancer Research, Eden Prairie, MN.
10. The TPA-dependent focus-forming phenotype of UV-TDTx1, UV-TDTx8, and UV-TDTx10 cultures was stable. Cells cultured for several passages in the presence or absence of TPA gave rise to secondary foci in the presence, but not the absence, of TPA. Moreover, the TPA-dependent focus-forming phenotype was reversible. When cells from TPA-dependent secondary foci were replated with C3H10T1/2 cells and grown in the absence of TPA, no foci occurred.

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12. C3H10T1/2 cells, in 20% serum, can suppress focus formation by some chemically induced C3H10T1/2 transformants. When grown in 5% serum, focus formation occurred in these mixed cultures of selected transformed C3H10T1/2 cells and C3H10T1/2 cells [J. S. Bertram, *Cancer Res.* **37**, 514 (1977)]. Cyclic adenosine 3′,5′ phosphodiesterase inhibitors greatly enhance suppression of focus formation in such mixed cultures [J. S. Bertram and M. B. Faletto, *Cancer Res.* **45**, 1946 (1985)].
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Inhibition of Secretion of Hepatitis B Surface Antigen by a Related Presurface Polypeptide

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The presurface (preS) proteins of hepatitis B virus are structural components of the viral envelope that may play important roles in virion assembly and infectivity. They are specified by a large open reading frame that includes the coding region for the major surface (S) protein in its 3′ half. Translation of the preS proteins initiates upstream from the S region, giving rise to proteins that are composed of the S domain and an additional 163 (preS1) or 55 (preS2) amino acids. Little is known about the biosynthesis and assembly of these proteins. The expression of the S and preS1 proteins was examined by transfecting cultured mammalian cells with viral DNA and injecting synthetic messenger RNA's into *Xenopus* oocytes. In contrast to the proteins encoded by the S region, the preS1 proteins are not detectably secreted into the culture medium. Furthermore, when the S and preS1 proteins are synthesized together, secretion of the S proteins is specifically and strongly inhibited. The results suggest a unique molecular interaction during secretion of the S and preS proteins that may be important for virus assembly.

A DISTINCTIVE FEATURE OF ACUTE or chronic hepatitis B virus (HBV) infection is the accumulation in the serum of large amounts of particulate hepatitis B surface antigen (HBsAg) (*1*). The major component of HBsAg particles is the product of the viral S gene, a protein of 226 amino acids that is present in nonglycosylated ($p24^S$) and glycosylated ($gp27^S$) forms (*2*). Two sets of less abundant HBsAg-related polypeptides were recently identified; these polypeptides are specified, in part, by the presurface (preS) region, a 522–base pair (bp) open reading frame (*3*) that is upstream and in phase with the S coding region. These larger proteins, termed the preS1 and preS2 proteins, are composed of the 226 residues encoded by the S region plus an additional 163 (preS1) or 55 (preS2) NH_2-terminal amino acids derived from the preS region (Fig. 1A) (*4, 5*). The function of the preS proteins is unknown, but they are present in greater abundance in virions than in the more numerous subviral HBsAg particles (*5*), suggesting a role for these protein domains in infectivity or virus assembly or both.

In order to examine HBV preS gene expression in mammalian cells, we first constructed two plasmids containing the HBV S and contiguous preS regions positioned downstream from the strong promoter in the long terminal repeat (LTR) of Rous sarcoma virus (RSV). These constructs, termed pSA10 and pSA41, differ only in the orientation of the HBV-specific insert relative to the LTR (Fig. 1A). Both plasmids are capable of directing the synthesis of the S and preS2 proteins by virtue of the HBV S gene promoter that lies within the preS1 open reading frame (*6, 7*); however, pSA41, unlike pSA10, can also promote the expression of the full preS1 reading frame from the RSV LTR. Mouse LTK^- cells, which are deficient in thymidine kinase, were transfected with these plasmids together with the thymidine kinase (TK) gene of herpes simplex virus, and TK^+ foci from each transfection were isolated. To confirm the presence of the predicted transcripts, we performed Northern blotting analysis of polyadenylated [poly(A)$^+$] RNA prepared from the two cell lines (Fig. 1B). Both cell lines exhibited the 2.2-kb message previously demonstrated to originate from the HBV S promoter (*6, 7*). In addition, pSA41 transformants had an abundant additional transcript of approximately 2.6 kb; this species originates from the RSV LTR, as shown by its annealing to probes from U5 but not U3 sequences of RSV, as well as to probes specific for the HBV preS1 region. We then tested the two cell lines for HBsAg expression by radioimmunoassay. As expected, pSA10 transfectants expressed HBsAg in the cytoplasm and in the culture medium (Fig. 1C). Surprisingly, however, cells transfected with pSA41, while expressing HBsAg in the cytoplasm, showed no detectable secretion of immunoreactive material into the medium.

To examine further the generality of this finding and to develop a system in which constructions could be rapidly screened without the necessity for selecting stable transformants, we turned to a transient expression system based upon SV40 transcription and replication signals (*8*). Briefly, plasmids containing the S (pSV24H) or the entire preS and S (pSV45H) coding regions cloned downstream from the SV40 early promoter (and *ori*) sequences (Fig. 2A) were constructed and used to transfect COS7 cells. These cells contain integrated SV40 genomes that supply T antigen in trans, resulting in the amplification of transfected DNA bearing the viral replication origin. Forty-eight hours after transfection of cultures with each construct, cell extracts and media were assayed for HBsAg by quantitative radioimmunoassay. As expected, cells transfected with pSV24H produced abundant quantities of HBsAg, which was readily demonstrable in both medium and cytoplasm (Fig. 2B). Parallel cultures similarly transfected with pSV45H synthesized comparable quantities of cytoplasmic antigen, but the levels of extracellular antigen were 1/12 to 1/10 those of the intracellular fraction.

These studies suggest that the preS1 anti-

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