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Molecular Basis for the Attack on Cancer

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ABSTRACT **Studies of cell transformation by RNA and DNA tumor viruses by DNA–RNA hybridization and extension of these techniques to analysis of human cancer tissue are reviewed. The molecular hybridization technique, it is suggested, will be useful in further studies of the role of oncogenic RNA and/or DNA viruses in causation of cancer.**

Until recently, the prospects for understanding the nature of cancer and for developing a rational basis to combat this disease were dim. But now we can be more optimistic, for we are rapidly acquiring the knowledge, techniques, experimental systems, and scientific insight to investigate and to understand the molecular basis of neoplasia and to effectively use the new knowledge for the prevention and cure of cancer. This optimism is based on spectacular advances in molecular and cell biology, and the more recent development of cell culture systems to study cell transformation and growth control in mammalian cells. I summarize below the recent progress in understanding cell transformation by DNA and RNA tumor viruses, and in evaluating the role of virus-specific and host-specific gene expression in human cancer.

Oncogenic viruses

DNA and RNA tumor viruses hold the key to the understanding of mammalian cell function and growth control in molecular terms and for investigation of the molecular biology of mammalian cells. Viruses afford some of the most powerful systems for analysis of DNA replication, and RNA transcription and translation in mammalian cells.

About 150 of 600 known animal viruses are oncogenic (Table 1). Oncogenic DNA viruses undergo two types of interaction with cells (1): (*i*) *productive infection*, in which hundreds of thousands of virus particles are assembled and the cell is killed; and (*ii*) *cell transformation*, in which virus does not replicate but the cell is altered morphologically, and cell multiplication and macromolecule synthesis are controlled in part by viral genes.

Among DNA tumor viruses, polyoma, SV40, and three groups of oncogenic human adenoviruses have been analyzed for the mechanism of viral replication and cell transformation (ref. 1; and see below); only herpesviruses have been associated with natural cancer in animals and man, e.g., Burkitt's lymphoma and nasopharyngeal carcinoma of humans see Klein, G. (1972) *Proc. Nat. Acad. Sci. U.S.A.* **69**, 1056–1064), RNA tumor viruses, e.g., leukemia–sarcoma viruses, induce cancer naturally in several animal species, and particles with properties similar to these viruses, i.e., 70S viral RNA and the presence of an RNA-directed (RNA→DNA) polymerase, have been detected recently in human milk (2) and in cultured human rhabdomyosarcoma cells (3). Whether these viruses infect humans or play a role in human cancer is not known.

Virus particles may not be a major cause of human cancer; however, the exciting possibility that cellular genes that contain information similar to that of RNA tumor viruses are expressed in human cancer, i.e., the oncogene hypothesis of Huebner and Todaro (4) (see Todaro, G. (1972) *Proc. Nat. Acad. Sci, USA* **69**, 1009–1015) is supported by the recent induction of endogenous RNA tumor-virus information in normal rodent and avian cells (5–7). If human cancer cells do indeed contain such information, it is possible that the hypothetical "human oncogene" will be expressed as a virus particle in a rare individual. Perhaps RD-114 virus, isolated from human rhabdomyosarcoma cells, is an example of this (3).

Mechanism of cell transformation by DNA tumor viruses

An overview of studies of the past 7 years (1) is given below, illustrated by data from investigations with human adenoviruses from our laboratory; similar findings are known for polyoma virus and SV40 (1).

Productive infection of human cells with human adenovirus

In this model for analysis of the synthesis of macromolecules in mammalian cells, viral DNA enters the nucleus, and like host DNA, is replicated and transcribed to polycistronic RNA molecules. These molecules are cleaved before entry into cytoplasmic polyribosomes, where they are translated to virus-coded proteins. Early viral RNA species, transcribed from 10–20% of each viral DNA strand by the cell DNA→RNA polymerase probably code for tumor antigens and other proteins involved in late-gene transcription and viral DNA replication (8); some of these proteins are involved in transformation of nonpermissive cells. Late in infection, viral

TABLE 1. *Oncogenic viruses*

I. DNA viruses (about 50): *Papillomaviruses:* man, dog, and cow; *Polyomaviruses:* polyoma virus (murine), SV40 (simian); *Adenoviruses:* 12 of 31 human, 6 simian, 2 avian, and bovine; *Herpesviruses:* Burkitt's lymphoma (human), Lucké carcinoma (frog), and Marek's disease (chicken).

II. RNA viruses (about 100): *Avian* leukemia-sarcoma viruses; *Murine* leukemia-sarcoma viruses; *Murine mammary* tumor viruses; *leukemia-sarcoma* viruses of cat, hamster, rat, and guinea pig; *Human milk* virus (?)*; *Human RD-114* virus (?)†

* Moore *et al.* (2).

† McAllister *et al.* (3).

Abbreviation: MSV, mouse sarcoma virus.

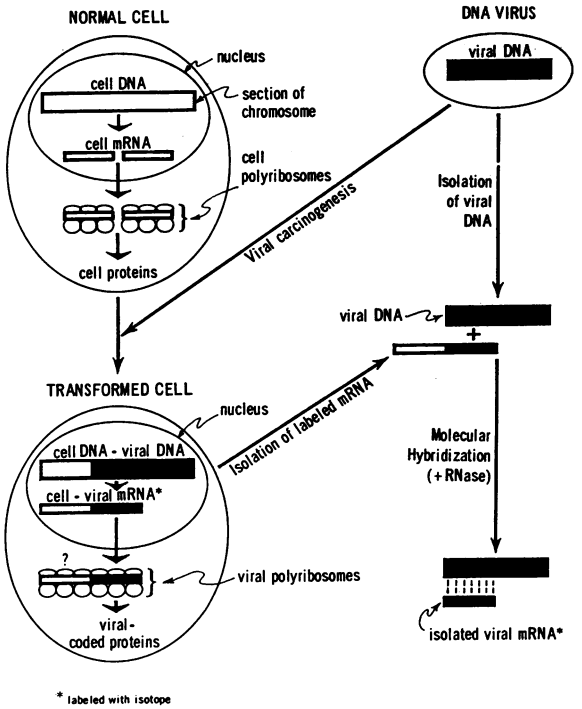

FIG. 1. Scheme depicting the mechanism of cell transformation by DNA viruses.

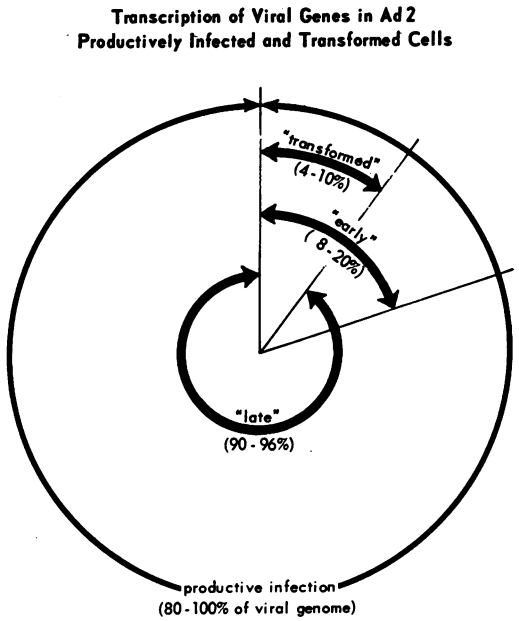

FIG. 2. Diagram representing the fractions of the viral genome transcribed in adenovirus (Ad) 2-infected and transformed cell.

RNA transcripts code for the 8–9 viral structural proteins and other proteins involved in maturation of progeny virus.

Cell transformation by human adenoviruses

Twelve of 31 human adenoviruses transform cells in culture, induce tumors, or both. Huebner *et al.* discovered in 1963 (9) that transformed cells, while they synthesize no virus, make virus-specific tumor antigens, also detected early during productive infection. This discovery provided the first clues that the viral genome may persist, and suggested further that a viral gene product expressed early in productive infection was expressed in transformed cells. The sequence of events in cell transformation, elaborated in the last 7 years, is diagrammed in Fig. 1. Evidence for the proposed steps is briefly described below.

Viral DNA Sequences in Transformed Cells. DNA from cells transformed by adenovirus 2, 7, or 12 contains multiple copies of viral DNA sequences that (*i*) hybridize with adenovirus complementary RNA (cRNA) (8) and (*ii*) increase the rate of reassociation of viral DNA strands (Table 2). Viral DNA may be associated with specific chromosomes, as suggested by cytological hybridizations with viral cRNA (Loni and Green, unpublished data).

TABLE 2. *Viral DNA sequences in cells transformed by human adenoviruses**

Cell	Viral DNA equivalents per cell: By hybridization with viral cRNA	Viral DNA equivalents per cell: By reassociation kinetics
Adenovirus 12-transformed hamster	53–60	22, 22
Adenovirus 7 hamster tumor	86–97	25, 27
Adenovirus 2-transformed rat (8617)	22–30	5, 7

* From Fujinaga and Green (unpublished data).

Transcription of Viral Genes in Transformed Cells. A large fraction of the mRNA fraction that is present in cytoplasmic polyribosomes of cells transformed by 10 human adenoviruses is virus-specific (8). These viral RNA sequences are transcribed from a few "early" viral genes, as shown by the results of extensive experiments on DNA–RNA hybridization–competition (Fig. 2) (8). Virus-specific RNA occurs in transformed cells as polycistronic RNA molecules. These molecules are transcribed from integrated viral DNA and contiguous cellular DNA, since they hybridize both with DNA from virus and from untransformed cells (Table 3). The high efficiency of hybridization with cellular DNA suggests that the cellular DNA sequences contiguous to viral DNA are highly reiterated (11).

Analysis of human cancers for genetic information of DNA tumor viruses by molecular hybridization

Cells transformed by DNA tumor viruses possess viral DNA sequences, virus-specific RNA sequences, and virus-specific

TABLE 3. *Hybridization of virus-specific RNA from hamster tumors that are induced by adenovirus 7, with both viral and cellular DNA**

Source of RNA	RNA input (cpm)	DNA: Source	DNA: μg/filter	Bound radioactivity: cpm	Bound radioactivity: %
Cells transformed by adenovirus 7	1180	Adenovirus 7	5	587	50
		Hamster cell	50	182	15.5
		Escherichia coli	50	28	2.4

* From Tsuei, Fujinaga, and Green (12).

TABLE 4. *Analyses of human cancers for genetic information of DNA tumor viruses by molecular hybridization and immunological analysis*

1. Hybridization of labeled tumor RNA with viral DNA.
2. Hybridization of tumor DNA with labeled complementary viral RNA.
3. Hybridization-competition for sites on viral DNA between tumor RNA and labeled virus-specific RNA derived from: (*a*) virus-infected cells, (*b*) virus-transformed cells, or (*c*) *in vitro* complementary RNA synthesized by *E. coli* RNA polymerase.
4. Immunological analysis of human tumors for virus-specific antigens or sera for virus-specific antibody.

tumor antigens (1). As described in Table 4, these virus-specific molecules may be detected readily by molecular hybridization and immunological analysis, thus providing a basis for analysis of the involvement of viruses in human cancers. Extensive analysis of sera from cancer patients for antibody directed against adenovirus tumor antigen were negative (summarized by McAllister, Gilden, and Green, manuscript in preparation). Molecular hybridization–competition analysis (Table 4, no. 3) is especially useful for screening large numbers of human tumors and provides a general approach for testing the 50 known DNA viruses as etiological agents of cancer or other diseases. By this procedure, 200 human cancers from 19 different types of organs were analyzed for base sequences specific for adenovirus 2, 7, and 12; the results were negative. For example (see Table 5), RNA from cancer of the liver, stomach, lung, breast, and ovary did not compete with viral [^{3}H]RNA from transformed cells (88–110% of control), while homologous viral RNA competed well (18–19% of control).

While they suggest that adenoviruses are not causes of human cancer, these tests do not eliminate this possibility. Although cells transformed by adenovirus in culture contain

TABLE 5. *Molecular hybridization tests for genetic material of adenovirus 12 in human cancers**

Completing unlabeled RNA	Competing RNA (μg)	Ad 12 [^{3}H]RNA (cpm × 10^6)	cpm Bound above background†	% Of control
1. Control (no RNA)	—	1.15	355	100
2. Adenovirus 12-transformed cell	150	1.15	64	18
3. KB (human cell culture)	300	1.15	361	102
4. Liver, undifferentiated carcinoma metastatic	277	1.15	334	94
5. Stomach, adenocarcinoma	170	1.15	363	102
6. Lung, bronchiolar carcinoma	274	1.15	375	106
7. Breast, adenocarcinoma	300	1.15	385	108
8. Ovary, granulosa cell carcinoma	294	1.15	374	106

* From Green, Devine, Thornton, and Pinkerton (unpublished data). Ad, adenovirus.

† Average of two separate experiments.

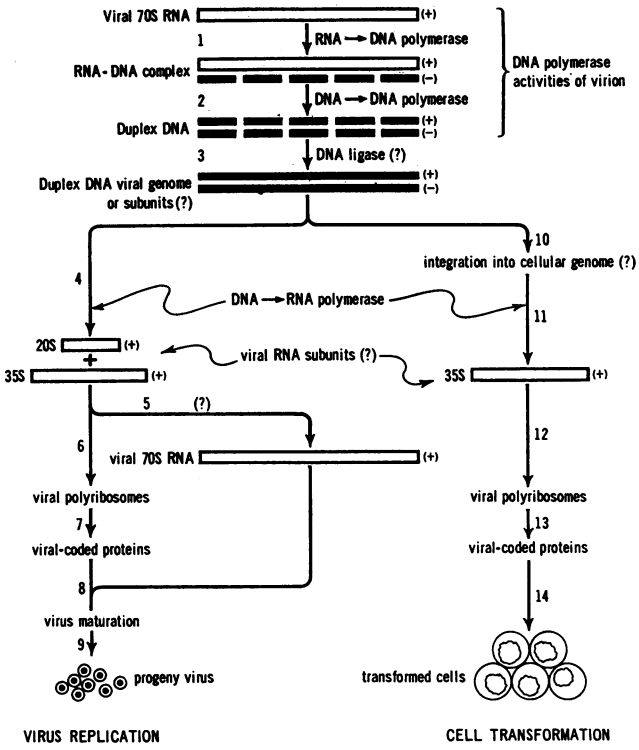

FIG. 3. Scheme of RNA tumor-virus DNA polymerase activities and their possible functions in virus replication and cell transformation.

about 10,000 viral RNA molecules, and the tests are sensitive enough to detect 1000 RNA molecules, fewer than 1000 viral RNA molecules might occur in human cancers. This possibility is real, and more sensitive hybridization tests with synthetic RNA specific for the adenoviruses, as well as for herpesvirus 1 and 2 (the latter is possibly involved in cervical cancer) are now in progress.

Biochemical mechanism of virus replication and cell transformation by RNA tumor viruses

The intense research activity subsequent to the recent discovery of the viral RNA→DNA polymerase (13, 14) suggests the tentative scheme of Fig. 3: The 70S viral genome is transcribed by the viral RNA→DNA polymerase [1] to small DNA pieces (15, 16) whose sequences are derived from most of the viral genome (ref. 17; Green, unpublished data). These may be further replicated [2] and assembled (perhaps by a DNA ligase) to form a viral DNA genome, or viral DNA subunits [3]. Viral DNA may be integrated in transformed cells [10], or even in virus-producing, nontransformed cells. Preliminary studies using cytological hybridization with the MSV DNA product suggest that viral DNA is localized in chromosomes of MSV-transformed cells (Loni and Green, unpublished data).

Virus Replication (*Fig. 3*, 4–9). Viral RNA sequences are present in molecules sedimenting at 35 and 20 S in cells in which MSV replicates (Tsuchida, Robin and Green, manuscript in preparation). These RNA sequences may be precursors to the viral genome [5] or messenger RNA for viral proteins, or both [6, 7] (Vecchio, Shanmugam, Tsuchida, Attardi, and Green, unpublished data). Assembly of mature virus from

TABLE 6. *Analysis of human cancers for genetic information of RNA tumor viruses*

1. Molecular hybridization between cancer RNA and labeled DNA prepared by the viral RNA→DNA polymerase.
2. Search for RNA→DNA polymerase activity in human cancers.
3. Induction of virus particles in human cancers by treatment with chemicals. Assay by RNA→DNA polymerase activity or by labeled particles using isopycnic centrifugation.
4. Search for human virus-specific antigens in human tumors.

viral proteins and viral RNA subunits probably occurs at the cell membrane [8, 9].

Cell Transformation (Fig. 3, 10–14). Cell line HT-1 contains the entire MSV viral genome, but synthesizes no recognizable viral proteins (18). HT-1 cells transcribe some 35S, but no 20S viral RNA. Thus, in cell transformation by RNA tumor viruses, as in transformation by DNA tumor viruses—such as adenoviruses—a portion of the viral genome is expressed, and probably maintains the properties of the transformed cell. The viral proteins coded for by viral 35S RNA in HT-1 cells are likely candidates for "transforming proteins."

Analysis of human cancers for genetic information of RNA tumor viruses

Current knowledge suggests several means for analysis of human tumors for virus-specific genetic information, as described in Table 6. One of the most powerful, sensitive, and specific methods is molecular hybridization. Every cell line infected with, or transformed by, murine RNA tumor viruses that we have examined possesses virus-specific RNA sequences readily detected by molecular hybridization (Fig. 4). Using the same basic test, described in Table 7, we have analyzed human tumors for base sequences that are specific for RNA tumor viruses, as illustrated in Table 8 with the DNA product of the feline sarcoma viruses. Thus far, no RNA that is specific for feline sarcoma virus has been detected in cancer of the liver, larynx, lung, ovary, breast, and cervix; 50 additional specimens from human cancers were also negative. Thus, the current concern that the feline sarcoma virus may be

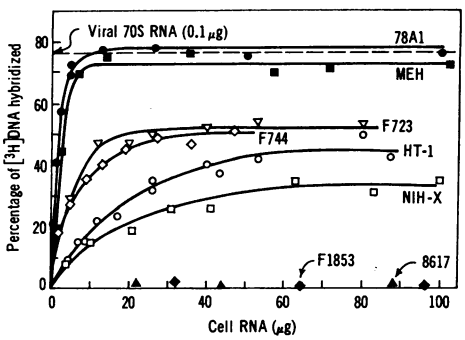

FIG. 4. Hybridization of the DNA product from mouse sarcoma virus (Moloney) with RNA from transformed cells that produce (78A1, MEH), or do not produce virus (HT-1, NIH-X F723, F744). F1853 and 8617 are normal and adenovirus 2-transformed rat cells, respectively.

TABLE 7. *Analyses of human cancers for genetic information of RNA tumor viruses by molecular hybridization*

1. *Hybridization reaction:*

   Human cancer RNA + [^{3}H]DNA* $\xrightarrow[\text{conditions}]{\text{annealing}}$ [^{3}H]DNA–RNA hybrid

2. *Quantitation:*

   Annealed product analyzed by hydroxyapatite chromatography or by Cs_2SO_4 density-gradient centrifugation.

* Prepared by copying viral RNA genome *in vitro* with the viral RNA→DNA polymerase.

involved in human cancer is probably unfounded. We have also tested the ability of RNA from human cancers to hybridize with the DNA products of the feline leukemia virus grown in human cancer cells, the Mason Pfizer monkey tumor, murine sarcoma virus, and the RD-114 virus. Positive hybridization with human cancers were found (Green, Richardson, Morrissey, and Pinkerton unpublished results). Spiegelman *et al.* (personal communication) have recently found that the DNA product of the mouse mammary tumor virus shares some sequences with RNA from human breast cancer. The significance of these observations for the etiology of human cancer is of great concern.

Summary statement

As described above, impressive progress has recently been made in understanding cell transformation by RNA and DNA tumor viruses in molecular terms, and in application of this knowledge to the analysis of human tumors for viral genetic information. For example, the powerful tools of molecular hybridization have permitted the analysis of human cancers for viral genetic information, by use of reagents developed from the studies of viral nucleic acid synthesis. The data from investigation of DNA tumor viruses, although negative thus far, emphasize the need for further refinement of the molecular hybridization technique in order to show or exclude the in-

TABLE 8. *Molecular hybridization tests for base sequences of feline sarcoma virus (FeSV) in human cancer RNA**

Source of RNA	[^{3}H]DNA cpm eluted with 0.12 M buffer	0.40 M buffer	% Duplex
Controls:			
Human KB cells	1244	33	2.6
FeSV RNA	809	371	31.5
Human cancer:			
Hepatoma	1234	31	2.5
Larynx	1243	40	3.1
Lung	1196	32	2.7
Liver	1271	35	2.7
Ovary	1392	32	2.3
Breast	1291	23	1.7
Cervix	1226	28	2.3

* Analysis of [^{3}H]DNA product hybridized with RNA and analyzed by hydroxyapatite chromatography (Green and Morrissey, unpublished data).

POLYMERASES OF ANIMAL VIRUSES

RDRP	RNA-dependent RNA polymerase
RDDP	RNA-dependent DNA polymerase
DDDP	DNA-dependent DNA polymerase
DDRP	DNA-dependent RNA polymerase

	Virus Group	Members: No.	Members: Example	Polymerase
RNA Viruses	Picornavirus	~200	Poliovirus	Induces RDRP
	Arbovirus	~200	Mengovirus	Induces RDRP
	Reovirus	3	Reovirus	Carries RDRP
	Myxovirus	5	Influenza	Carries RDRP
	Paramyxovirus	13	Newcastle Disease	Carries RDRP
	Rhabdovirus	2	Vesicular stomatitis	Carries RDRP
	Leukemia-sarcoma viruses	~100	Rous sarcoma, Human breast milk virus	Carries RDDP
DNA Viruses	Parvovirus	8	Kilham rat	Carries DDDP
	Polyomavirus	3	Simian virus 40	Induces DDDP
	Papillomavirus	5	Shope papilloma	Induces DDDP
	Adenovirus	55	Human adenovirus type 12	Induces DDDP
	Herpesvirus	12	Herpes simplex	Induces DDDP
	Poxvirus	24	Smallpox	Induces DDDP and carries DDRP

FIG. 5. Functions of DNA and RNA polymerases from members of 13 groups of animal viruses.

volvement of a small amount of virus-specific sequences from human and animal pathogens in the etiology of cancer. The first positive results in hybridization of human cancer RNA with the DNA product of RNA tumor viruses opens up the possibility that, as suggested by the oncogene hypothesis, specific gene sequences common to viruses and human cells could be involved in cancer. In this regard, the possible isolation of "human RNA tumor viruses" from those rare individuals in which "oncogene" information may be expressed as virus is of great importance, for it would provide the opportunity to test the role of this human genetic material in cancer by molecular hybridization of cancer RNA with the DNA product of the "human virus." In addition, the macromolecular components of this possible "human virus" would provide the reagents to analyze the regulation of "cancer specific" information using subcellular fractions derived from human cells as experimental systems.

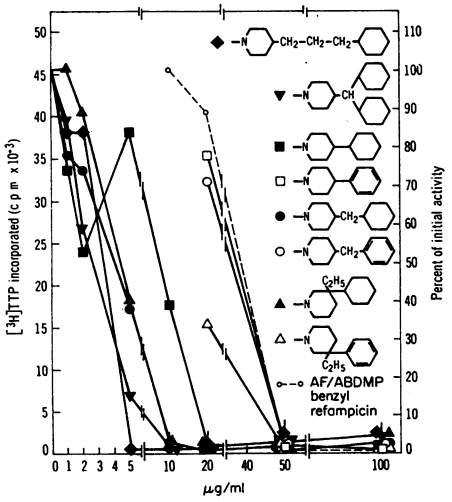

FIG. 6. Inhibition of DNA → DNA polymerase of MSV (Harvey strain) by 3-cyclic amine derivatives of rifamycin SV that contains cyclohexyl substituents.

An example of how basic information can be applied to chemotherapy is provided by studies of virus replication in bacteria and in eukaryotic cells that indicate that the specificity of polymerase molecules plays a major role in the control of DNA replication and gene expression. The specific polymerase requirements for the replication of animal viruses is most illuminating. As shown in Fig. 5, members of each of the 13 animal-virus groups either possess RNA or DNA polymerase or an increase in polymerase activity in cells. Perhaps the chemotherapy of viral diseases and of cancer can be based on the selective inhibition of specific polymerase molecules. Until recently, rifamycin SV derivatives were known to inhibit bacterial RNA→DNA polymerases, but not polymerase of mammalian cells (19). However, modification of rifamycin SV (20) by introduction of substituents such as cyclohexyl 3-cyclic amines at position 3 of the ansa ring produces strong inhibitors of DNA polymerase of RNA tumor virus (Fig. 6) and mammalian cells (21), and inhibits cell transformation by RNA tumor viruses (21). But, if inhibitors are to block virus-specific or cancer-specific information, they must be directed to the specific recognition sites on the polymerase. Viral and cellular polymerases do have relevant structural differences, as shown by their different template specificities and cellular functions. The systematic screening of polymerase inhibitors, such as rifamycin derivatives, and the increased understanding of polymerase reactions may help in the development of inhibitors of specific polymerases that possess the properties desired for the control of specific gene expression.

Unfortunately, further progress is often limited because we know only the bare outlines of the critical processes involved in growth control in normal and cancerous human cells. For example, a major unsolved problem is how proteins that are coded by a virus in cells transformed by RNA and DNA tumor viruses induce and maintain the transformed properties of the cell. Although the isolation of these proteins from transformed cells may be achieved, identification of their functions in cell transformation and control of these functions will almost certainly be more difficult until we supplement our meager knowledge of the synthesis and regulation of macromolecules in mammalian cells.

A comprehensive investigation of the molecular biology of the human cells is required in which some of the most useful systems will be *in vitro* preparations that replicate viral DNA, and transcribe and translate viral RNA. The success of this new program will depend upon extensive participation of present and future biological scientists in large multidisciplinary programs at many universities. Government support similar to research contracts, but with longer financial commitments, may be required to provide the flexible and large-scale funds needed for expensive laboratories that specialize in cell culture and virus research. The potential accomplishments of these investigations—both intellectually, in understanding the functions of the complex mammalian cell, and practically, in its applications to human cancers and other diseases—may provide the most significant scientific achievements of the 20th century.

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