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*THE NATURE AND LOCALIZATION OF THE SV40-INDUCED COMPLEMENT-FIXING ANTIGEN**

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Neoplastic transformation of mammalian cells by a number of oncogenic viruses is accompanied by the appearance of new "cellular" antigens. These are detectable by their ability to induce resistance to tumor implantation in susceptible animals[1] or by their ability to fix complement in combination with serum from tumor-bearing animals.[2–5] The relationship between the transplantation antigen and the complement-fixing (CF) antigen remains unknown; however, considerable evidence in the case of SV40 and polyoma viruses indicates that they are distinct from overt antigens of the intact virus.[3, 5] Indeed, the new antigens persist, apparently indefinitely, as the sole evidence of an original infection after virus can no longer be detected.

The SV40-induced CF antigen (SV40ICFA) is virus-specific and not species-specific; i.e., serum from hamsters bearing SV40 tumors reacts specifically with cells of different species transformed by SV40 virus.[3, 4] (In order to allow for the possibility that the antigens demonstrated by the complement-fixing and transplantation tests are distinct, the former is identified as SV40ICFA and the latter as SV40ITA.) It would appear, therefore, that synthesis of this antigen requires the persistence of at least some part of the SV40 genome. One might thus presume that SV40ICFA represents an "internal" virus antigen, a viral precursor, or a new nonviral protein coded for by the viral genome. Although our results to date favor the last of these possibilities, there is as yet no evidence bearing on the biological function of this antigen.

Materials and Methods.—Cells: The SV40-transformed human cells were the lines designated

W18 Va2 and WI26 Va4.[6] These cells have been virus-free for over $1^1/_2$ years and were known to contain SV40ICFA.[4]

The SV40 hamster transformed cells originated from a line established by SV40 infection of primary hamster embryo fibroblasts. The line has been carried for over 3 years both as *in vitro* cultures and as tumors in animals and is virus-free.[7]

CF tests: All tests were carried out by a microtechnique[8] using 1.6–2.0 exact units of complement and fixation overnight at 5°C. Antigen preparations generally consisted of supernatants of 10% v:v suspensions of frozen-thawed cells.

Immune sera: Several immune sera were used in these studies: (*a*) pooled sera from hamsters bearing SV40-induced transplantable tumors, with no detectable neutralizing antibodies against SV40;[9] this pool fixed complement in the presence of optimal dilutions of SV40 tumor extracts at a dilution of 1:160; (*b*) pooled sera obtained from hamsters injected with SV40ICFA-positive extracts of hamster tumors; this pool had a titer of 1:80; (*c*) pooled sera from hamsters bearing polyoma-induced transplantable tumors, with no neutralizing activity against polyoma virus; this pool fixed complement in the presence of polyoma tumor extracts at a dilution of 1:32.

Immunofluorescence: Fluorescent antibody staining was carried out by the indirect method. Coverslips were air-dried and fixed in cold acetone or acetone-alcohol (2:1) for 10 min. Hamster immune serum was then overlaid, and the coverslips were incubated at 37° for 45 min. Following three rinses with buffered saline, the coverslips were blotted dry and then overlaid with rabbit anti-hamster globulin labeled with fluorescein isothiocyanate (Baltimore Biological Products). Following further incubation, 45 min at 37°, the coverslips were rinsed and mounted in buffered elvanol[10] and then examined using a Reichert ultraviolet microscope with a dark field condensor. Rabbit fluorescein-labeled anticomplement was obtained from Dr. Werner Henle, Children's Hospital, Philadelphia. Staining for viral antigen was carried out directly using labeled anti-SV40 serum.[9]

Sucrose gradients: Linear sucrose gradients of approximately 5–20% were prepared with the aid of a mixing device. Estimations of sedimentation constant and molecular weight were made[11] on samples collected by puncturing the bottom of gradient tubes. Gradients contained bromphenol blue-labeled bovine serum albumin (*ca.* 4.5S and MW 70,000) as a marker.

Enzymes: Trypsin (2× crystallized salt-free, Nutritional Biochemicals) was used at 0.5 mg/ml. DNase (2× crystallized, Worthington) was used in concentrations to 250 μg/ml in saline supplemented with 5×10^{-3} *M* Mg^{++}. RNase (2× crystallized, Worthington) and 5× crystallized (Nutritional Biochemicals) was incubated in a boiling water bath for 10 min before use. RNase was used in concentrations of 25–200 μg/ml. Incubations with SV40ICFA preparations were generally carried out for 2 hr at 37°C. Trypsin activity was stopped by the addition of egg white inhibitor at twice the trypsin concentration.

Immunoprecipitation of tritiated uridine-labeled extracts: Human transformed cells (W18 Va2) were incubated with tritiated uridine (15 μc/ml; 3.2 C/mM) for 1 hr. After the incubation period the cells were washed with phosphate buffered saline (PBS) and then freeze-thawed several times. The resulting suspension was centrifuged three times at 30,000 *g* for 1 hr, the sediment being discarded each time. Aliquots of the supernatant were subjected to direct precipitation or indirect precipitation using rabbit antihamster serum to precipitate complexes of hamster antibody and SV40ICFA. Precipitates were collected under vacuum on glass-fiber filters (Gelman, type E). Filters were washed with 25 vol of cold buffer before radioactivity was determined in a liquid scintillation counter (Packard Tricarb).

Results.—Localization of SV40ICFA: Specific staining by the indirect immunofluorescent technique was observed in the nucleus of 100 per cent of the transformed human and hamster cells. Staining was especially brilliant in the human transformed cell lines, W18 Va2 and WI26 Va4 (Fig. 1). The cells have remained positive over a period of several months, during which at least 240 cell generations occurred. Staining areas were generally granular in appearance and distributed throughout the entire nucleus, although some instances of more homogeneous staining were observed. Cells in mitosis retained antigen which was clearly excluded from the region of chromosome metaphase plates. Nucleoli were always

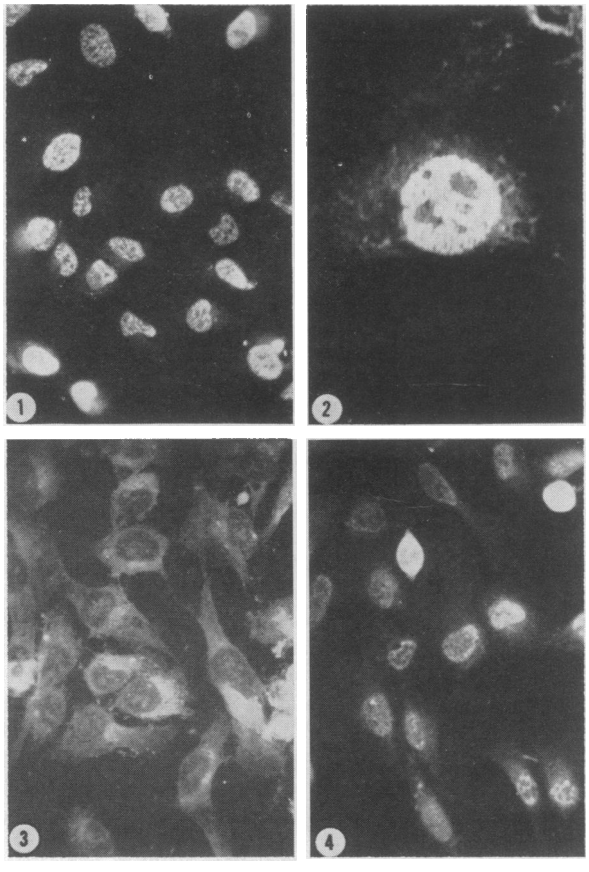

FIG. 1.—Human SV40-transformed cell line W18 Va2 (159th passage), stained with serum of tumor-bearing hamsters. Indirect method. Magnification ×240. Every nucleus is stained.

FIG. 2.—Hamster SV40-transplanted tumor at the 20th tissue culture passage, stained with serum of tumor-bearing hamsters. Indirect method. Magnification × 720. The cell nucleus is brilliantly fluorescent; however, the nucleoli are completely unstained.

FIG. 3.—Human SV40-transformed cell line W18 Va2 (159th passage in tissue culture) with serum of polyoma tumor-bearing hamsters. Indirect method. Magnification × 240. This picture was overexposed relative to Fig. 1 to show cellular outlines. All nuclei are completely unstained.

FIG. 4.—Human transformed cell line W18 Va2 (159th passage in tissue culture) treated with sera of tumor-bearing hamsters and guinea pig complement, and stained with fluorescent anti-complement. Magnification × 240. Every nucleus is stained in a pattern identical to Figs. 1 and 2.

clearly negative (Fig. 2). Similar results have been obtained for several other species of SV40-transformed cells, including hamster, by Pope and Rowe.[12]

Controls for antihamster and anticomplement staining included normal hamster serum, serum from animals bearing polyoma virus-induced tumors and normal saline. These controls have always been completely negative (Fig. 3). In addition, prior absorption of the positive hamster serum with human SV40ICFA preparations completely eliminated staining.

To test the identity of SV40ICFA, as detected in homogenates, with the nuclear staining material, slides were incubated with hamster positive serum and complement for 45 min at 37°. The slides were then washed and incubated with fluores-

cent anticomplement. Positive staining was observed which was indistinguishable in every feature from that observed with the indirect method, thus establishing the nuclear-staining material as SV40ICFA (Fig. 4).

Identical results have been obtained, using the indirect method, with over ten individual sera from hamsters either bearing tumors or immunized with hamster SV40ICFA.

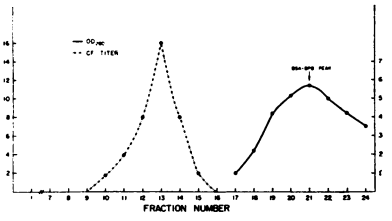

FIG. 5.—Sucrose gradient centrifugation of SV40ICFA from human cells. Centrifugation for 4 hr at 36,000 rpm in the SW39 rotor of the Spinco L; 0.5 ml of antigen preparation containing BPB-BSA was layered on a 5–20% linear sucrose gradient containing buffered saline. The CF peak has sedimented approximately 4 × farther than the BPB-BSA marker.

Properties of SV40ICFA: As obtained from tumors or tissue cultures the antigen is soluble in PBS and is found largely in the supernatant after 1 hr of centrifugation at 50,000 rpm in the #50 rotor of the Spinco model L ultracentrifuge (Table 1). In 5–20 per cent linear sucrose gradients the peak of CF activity sedimented approximately four times faster than the bromphenol blue-labeled bovine serum albumin used as a marker (Fig. 5). On this basis the sedimentation coefficient is approximately 18S, indicating a relatively high molecular weight, *ca.* 600,000.[11] It is possible that the high molecular weight results from association or aggregation of a number of identical subunits.

The antigen is insoluble in ammonium sulfate at half-saturation and is also precipitated at pH 4.5 (Table 1). While complete recovery is readily obtained upon resolution from ammonium sulfate, a severalfold loss of activity occurred on redissolving the precipitates and readjustment to neutrality. The maintenance of activity after ammonium sulfate precipitation provides a relatively easy and rapid method for preparing concentrated preparations of SV40ICFA for immunization studies, and also a means for storing antigen preparations over periods of a few months.

One of the prime difficulties encountered thus far has been the relative instability

TABLE 1

EFFECT OF VARIOUS TREATMENTS ON SV40ICFA

Sample*	Titer	Fluorescent antibodies
W18 Va2 −10% suspension	1/64	Positive
Solubility		
Supernatant of 3000 rpm	1/64	
50,000 (Spinco) 1 hr supernatant	1/32	
Pellet of above	1/2–1/4	
Ammonium sulfate precipitate (1/2 saturated)	1/64	
Ammonium sulfate supernatant	<1/2	
pH 4.5 ppt	1/8	
pH 4.5 supernatant	<1/2	
Thermostability		
6 hr 37°	1/8	
18 hr 37°	<1/2	
Enzyme treatment		
DNase (10–250 γ/ml)	1/64	Positive
RNase (25–200 γ/ml)	1/64	Negative
Trypsin (500 γ/ml)	<1/2	Negative

* All tests carried out with 8 units of antiserum.

of the antigen preparations. Whereas activity is gradually lost upon storage at 5° over a period of several weeks, incubation at 37° results in complete inactivation by 18 hr and significant inactivation after 6 hr (Table 1). While this relative lability may be due to enzymatic degradation, similar results have also been obtained with sucrose gradient purified material.

The antigen in tumor extracts would not adsorb to DEAE-cellulose columns at pH 6.5 and low ionic strength (0.0175 *M* phosphate). Gel filtration on Sephadex G-100 and G-200 resulted in recovery of antigen in the column-void-volume indicating molecular weight greater than 200,000, which is in agreement with the sedimentation estimate.

Enzyme treatments: The results of enzymatic treatment were followed wherever feasible by both immunofluorescent staining and CF tests (Table 1). Concentrations of DNase reaching 250 μg/ml had no effect in either system, nor was any marked effect seen with RNase in CF tests. Trypsin completely inhibited the reactivity of the CF antigen as did, surprisingly, RNase in the immunofluorescent test. The elimination of fluorescent staining by RNase has been observed consistently. Addition of trypsin inhibitor to the RNase did not prevent activity, while heparin, an inhibitor of RNase activity, did prevent the destructive effect. The discrepancy between the CF and immunofluorescence results was resolved by the demonstration of the CF antigen in the supernatant of fixed cells treated with RNase. These results suggested that the antigen is a protein closely associated with nuclear RNA in transformed cells. The RNase treatment presumably acts by solubilizing the complex without destroying antigenicity.

Immunoprecipitation: Further evidence of such association was found in immunoprecipitation experiments using extracts of cells labeled with tritiated uridine (Table 2). Using the indirect method, a severalfold greater amount of radioactivity was found in precipitates formed in the mixtures containing CF positive sera when compared to CF negative sera. The relatively low level of nonspecific activity found in the large precipitates allowed the performance of direct precipitation with the expectation that nonspecific trapping in the specific microprecipitates would be minimal. The serum volume was increased threefold in the direct method in an effort to ensure antibody excess. The results showed a tenfold difference in bound radioactivity in favor of the CF positive sera. Further,

TABLE 2

IMMUNOPRECIPITATION OF H^3-URIDINE-LABELED SV40ICFA-CONTAINING EXTRACTS

		Cpm of precipitate‡
A.	Indirect*	
	CF positive serum	2180; 2260
	CF negative serum	620; 558
B.	Direct†	
	CF positive	7346; 8448; 7639
	5% cold TCA wash	5873; 8458
	5% hot TCA wash	72; 61
	CF negative	
	(*a*) antipolyoma	1101; 576; 633
	(*b*) normal hamster	747; 601; 662

* 0.1 ml extract and 0.1 ml antibody (1:10 dilution) and 0.3 ml tris buffered saline were incubated at 37° for 30 min. Then 0.5 ml rabbit antihamster serum was added, and the mixtures were incubated for a further 60 min. Precipitates were processed as described under *Methods*.

† 0.1 ml extract and 0.3 ml antibody (1:10 dilution) were incubated at 37° for 2 hr and then at 5° for an additional 2 hr. Mixtures were then processed as described under *Methods*.

‡ Each figure represents a separate incubation.

this activity was retained after washing with 5 per cent cold trichloroacetic acid (TCA) but could be removed by 5 per cent hot TCA. This indicates that the radioactivity was in the form of macromolecular RNA.

It was not, however, possible to localize this activity with the CF peak in sucrose gradients despite several trials. This indicates again that the RNA component is not essential for antigenicity and may be separated from the antigen during sucrose gradient centrifugation.

Relationship to viral antigen: The human transformed cells used in this work have consistently been found negative for infectious virus. Attempts to induce virus or viral antigen in these cells by a variety of treatments successful in inducing phage production in lysogenized bacteria (mitomycin, UV, X ray) have been unsuccessful.[13] These cells did not react with high titer SV40-neutralizing antibody either in direct fluorescent experiments or in CF tests. On the other hand, the SV40ICFA immune sera contained no neutralizing antibody for SV40 and did not react in CF tests with the virus. Thus, as described previously,[3] the SV40ICFA is clearly distinct from overt viral antigens. However, recent observations[12] show clearly that SV40ICFA appears during an infectious cycle and is thus presumably not an exclusive characteristic of transformed cells.

The possibility that SV40ICFA represents a viral subunit of different antigenicity than the intact virus was tested, using methods similar to those used successfully for poliomyelitis virus.[14] Purified virus, 1–5 $\times$ 10^8 PFU/ml, prepared by cesium chloride density gradient centrifugation, was treated with 6 *M* guanidine-HCl for 4–18 hr at 37°. The guanidine was then removed by dialysis. This treatment did not "release" SV40ICFA. In addition, rabbits immunized with guanidine-degraded virus failed to produce antibodies reactive with transformed cells. Treatment with 8 *M* urea and prolonged dialysis versus high pH buffers also failed to yield SV40ICFA reactivity from intact virus. These results indicate that the antigen is not a viral precursor or an internal viral component.

Production of SV40ICFA during an infection cycle: African green monkey kidney (GMK) cells were tested in parallel by CF tests and immunofluorescence for the production of SV40ICFA, viral antigen, and infectious virus[15] at various times after infection. The results of a large number of experiments, summarized in Table 3, showed that SV40ICFA appeared at least 6–10 hr before the first indication of positive viral antigen staining. The first increase in infectious virus was generally observed 26–30 hr post infection. Similar results have recently been obtained by Rapp *et al.*[16]

Since 5-fluorodeoxyuridine (FUdR) had been shown to prevent SV40 replication and viral antigen synthesis,[17] the effect of this drug on SV40ICFA synthesis was studied. Prevention of viral replication and viral antigen immunofluorescence was

TABLE 3

APPEARANCE OF SV40ICFA AND VIRAL ANTIGENS DURING AN INFECTIOUS CYCLE

	SV40ICFA	Viral antigen	Infectious virus*
First appearance†	18 hr (1–10%)	24 hr (1%)	26–30 hr
Maximum†	48–60 hr (100%)	72 hr (60–90%)	72 hr

* Determined by plaque formation.
† Antigens detected by immunofluorescence. % positive cells shown in parentheses.

reproducibly achieved with FUdR at concentrations of 25–50 μg/ml present in the medium from 2 hr before infection and throughout the incubation period. Under these conditions, SV40ICFA production was, within a factor of two, the same as in control cultures (Table 4). The same results were obtained with cultures in which the FUdR was replaced every 12 hr. These results clearly imply that synthesis of SV40ICFA is under control of the incoming viral DNA while some DNA replication is necessary for the synthesis of viral coat antigen.

Discussion.—The evidence obtained in this work indicates that the SV40 cellular CF antigen (SV40ICFA) is a soluble protein, of relatively high molecular weight (*ca.* 600,000) associated with nuclear RNA, from which it can be dissociated by RNase. It is exclusively localized in 100 per cent of the nuclei of all the human and hamster SV40-transformed lines that have been tested. No specific nuclear zone (membrane or perinuclear) appears to be a preferred site, and it is absent from the nucleolus area and from the condensed chromosomes of the mitotic plates. When the nuclear membrane disappears during mitosis, it diffuses throughout the cytoplasm, to reassemble again in the nucleus at the end of the telophase. To our knowledge, there are no other examples of antigenic substances with this distribution, except for a protein nuclear antigen that reacts with sera from lupus erythematosus patients.[18]

The specific relationship of the antigen to SV40 virus is based on its absence from any other normal or polyoma virus transformed cells that have been tested and on its presence in cells from several species that have been infected with or transformed by SV40.[3, 4, 12]

In regard to the origin and function of SV40ICFA, the data are insufficient to formulate a clear hypothesis. The antigen does not seem to be a constituent of the viral coat, since antiserum against it does not react with complete SV40, nor does SV40 antiserum react with cell extracts positive for SV40ICFA. Furthermore, it is present in considerable concentration, as judged by fluorescent antibody staining, in cells that do not produce infectious virus and from which infectious virus or viral antigen cannot be recovered after several types of treatment. The failure to obtain any positive reactions of antiserum with chemically degraded virus, and the high molecular weight tentatively assigned to the antigen makes it improbable that SV40ICFA represents a viral subunit antigenically different from intact virus.

While the fact that the antigen appears earlier than viral antigen in a completely

TABLE 4

EFFECT OF 5-FLUORODEOXYURIDINE (FUdR) ON SYNTHESIS OF SV40ICFA, VIRAL ANTIGEN, AND INFECTIOUS VIRUS IN GMK CELLS

	SV40ICFA		Viral Antigen	
	CF titer (reciprocal)	Immunofluorescence, % of cells positive	Immunofluorescence, % of cells positive	Virus titer (PFU/ml)
Controls[a]	16, ≥16, ≥16, 4, ≥16	30–100%	60–90%	$10^{6.8}$, $10^{7.2}$
FUdR 25 γ/ml[b]	8, 8	50%	0[d]	$10^{2.7}$, $10^{3.7}$
FUdR 50 γ/ml[c]	8, 8, 8, ≥16, ≥16	20–80%	0[d]	$10^{2.2}$
FUdR 50 γ/ml[e]	≥16	—	—	—

[a] Samples from different experiments harvested between 52–74 hr post infection.
[b] FUdR present in the medium from 2 hr prior to infection until harvest.
[c] All CF tests with 8 units of serum. Under these conditions, titers of 1/16 or greater are correlated with 75–100% of cells showing positive fluorescence.
[d] No cells showing definite fluorescence on an 11 × 22 coverslip.
[e] FUdR replaced at 12-hr intervals up to 36 hr post infection.

infectious cycle tends to support the precursor hypothesis, the complete dissociation of SV40ICFA and viral antigen synthesis by 5-fluorodeoxyuridine is perhaps the strongest available evidence against this hypothesis. The necessity for DNA synthesis to occur before viral coat antigens can be synthesized has also been found in adenovirus-infected HeLa cells.[19] However, in other cases, viral protein synthesis occurs in the absence of DNA synthesis,[20, 21] and empty particles have been found in abundance in polyoma-infected cells treated with FUdR.[20] In the latter case, the concentration of FUdR employed was 20-fold lower than in our experiments. Concentrations of the inhibitor are critical, as noted by Melnick *et al.*[17] Thus, with SV40, levels of FUdR which reduced virus titers to less than 1 per cent that of controls still permitted significant synthesis of viral antigen to occur, while higher drug levels completely prevented viral antigen synthesis.

One of the other hypotheses then to be considered is that the antigen is a cellular product whose synthesis is activated by the viral infection and persists even in the absence of replication of infectious virus. However, it would seem improbable that such a cellular product would not be found even in minimal quantity in normal or other transformed cells. It is also unlikely that such a cellular product would be antigenically similar in all species tested. Nonetheless, analysis in detail of the antigen obtained from several sources will be necessary before rejection or acceptance of this hypothesis.

The most probable hypothesis is that SV40ICFA is a product coded by a persistent viral genome (or part thereof) that is not incorporated in the virion. It may be an enzyme related to the duplication of the viral genome or some other protein whose function is unknown at the present time. Arrest of virus maturation at any of several possible steps could account for retention of antigen, compatible with cell survival. Analysis of the purified antigen in terms of its chemical composition and possible biological activity and of the associated RNA component should help in defining any of these different possibilities. Experiments with this scope are now in progress.

Perhaps the major question concerns the relationship of the antigen to the process and maintenance of transformation itself. We have noticed considerable variation in antigen content in a number of cell lines transformed by SV40. For example, the human line W18 Va2 gives higher titers than WI26 Va4 for equal cell numbers and both in turn contain more antigen than the hamster transformed cells. This implies that the exact amount of antigen is irrelevant and is perhaps of significance only in indicating a persistent viral genome.

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*A DIFFERENTIAL EQUATION FOR THE THETA FUNCTION**

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The algebraic and transformation properties of the famous theta function,

$$\theta(z) = \sum_{n=-\infty}^{\infty} e^{i\pi n^2 z}, \quad Im(z) > 0,$$

have long been known. In this note we present an algebraic differential equation satisfied by $\theta(z)$.

Let n be an integer greater than 1, and k a nonzero complex number. For sufficiently differentiable f define the nonlinear differential operators

$$D_{n,k}(f) = \left(\frac{k}{1-n}\right) f^{(kn+n-1)/k} \frac{d^n}{dz^n} (f^{(1-n)/k}).$$

THEOREM. *$\theta(z)$ satisfies the differential equation*

$$(D_{3,1/2}(f))^2 + 32(D_{2,1/2}(f))^3 + \pi^2 f^{10}(D_{2,1/2}(f))^2 = 0.$$

Let H denote the set of complex numbers with positive imaginary part, and denote by Γ the group of analytic automorphisms of H generated by $z \to z + 2$ and $z \to -z^{-1}$. The proof of the theorem rests on the fact that $\theta(z)$ is an analytic automorphic form (with multiplier system) for Γ. The well-known transformation equations of $\theta(z)$ on the generators of Γ are

$$\theta(z) = \theta(z+2), \quad \text{and} \quad \theta(-z^{-1}) = (-iz)^{1/2}\theta(z),$$

where $i^2 = -1$ and the branch of the square root is taken which is positive for positive imaginary z.

The idea of the proof is to construct a linearly dependent collection of expressions