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Biological activity of the intact and cleaved DNA of the simian adenovirus 7

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ABSTRACT

Only the deproteinized DNA preparations of the simian adenovirus of the type 7 (SA 7) exhibited transforming and tumorigenic activity. The complex of the SA7 DNA with terminal protein (TP) did not exhibit either transforming or tumorigenic activity in cell cultures. In contrast to the transforming potential the infectious titers of the DNA - TP complex for the monkey kidney cells were 30-50 times higher than those of pure DNA. Cleavage of the SA7 DNA by specific endonucleases enhanced the tumorigenic potential of pure DNA, suppressed its infectivity and did not affect the lack of transformation capacity of the DNA - TP complex. The onc-gene was localized in the left terminal fragment with the minimal size $4,3 \times 10^6$D in the case of R.Sal I. The tumorigenic activity was found to decrease with an increase in the size of the DNA fragment containing the onc-gene.

INTRODUCTION

SA7 exhibits high oncogenic and transforming activities[1,2] which are manifested in experiments with intact virus as well as in experiments with isolated DNA[3,4]. The restriction[5] and physical[6] mapping of the SA7 DNA provided wide-ranging possibilities for localization of the DNA part which contains the onc-gene[7]. At the same time, DNAs of many adenoviruses including SA7 can be isolated from the virions[5,8] or from the infected cells[9] in the form of the DNA - protein complex which is found in solution primarily in the form of a noncovalently closed ring. The TP presumably linked covalently to the 5' ends of the duplex markedly enhances the infectivity of the isolated DNA preparations[4] but its properties are yet unknown.

This paper describes the simultaneous studies of the in-

fectivity and the transforming and oncogenic activities of the DNA and the DNA - TP complex both in the intact form and after cleavage with various restrictases. The preliminary results of these studies were reported at the VI-the USSR-USA Symposium on the oncogenic viruses[7].

MATERIALS AND METHODS

The SA7 virus was propagated in monkey kidney cells[10]. The virus was purified by treatment with Freon-113 and centrifugation in cesium chloride density gradient[11]. The SA7 DNA was extracted by detergent-phenol deproteinization technique and pronase treatment[12]. The techniques for isolation of the endonucleases and cleavage of DNA were described elsewhere[13].

The DNA fragments produced by cleavage with restriction endonucleases were separated by electrophoresis in 1% agarose gel. The positions of DNA fragments were determined by staining the gels with ethidium bromide and exposure to the UV light. The gel bands with individual fragments were cut off, agarose was dissolved in saturated KI solution[14] and the DNA was concentrated and purified by column chromatography on hydroxyl apatite[15].

The DNA - TP complex was extracted from the purified SA7 suspension by treatment with guanidine chloride and subsequent gel filtration through sepharose 4B in the presence of guanidine chloride[16]. The DNA - TP complex was identified either by electron microscopy or by electrophoresis in agarose gel where the complex is found at the top of the gel[17].

Cell cultures and media. Primary monkey kidney cells were grown in 50-ml flasks to continuous monolayers in medium 199 containing 10% bovine serum and antibiotics.

Titration of the DNA and DNA - TP complex was carried out as described by Graham[18]. Prior to infection the cell monolayer was washed with medium 199 and fed with 5 ml of the BME containing 2% calf serum, and 0.5 ml of the mixture of the DNA solution with Ca^{++} was added to each flask. Two to four flasks with monkey kidney cell culture were used for each dilution of DNA or DNA - TP complex. The system was in-

cubated for 4 hours at 37°C. Then the mixture was poured off from the flasks, the cells were treated with 20% dimethylsulphoxide (DMSO) supplied by Serva (FRG) for 6 min, washed with medium 199 containing antibiotics and overlaid with 5 ml of 1.6% agar solution (Difco) prepared from Earl's solution supplemented with vitamins, amino acids and 10% calf embryo serum. After 4-6 days another 3 ml of agar containing 0.01% neutral red were added. The plaques were counted starting from the sixth day of incubation.

The transformation experiments were carried out with the primary cell culture (PC) of kidneys of the 5-7 days old WAG rats and fibroblasts (FRE) of the WAG rat embryos in the first subpassage. The cell cultures were grown in 50 ml dishes with a seeding density of 180×10^3 cells/ml for the PC and 150×10^3 cells/ml for the FRE. The cells were grown in medium 199 with the addition of 10% calf serum. The transformation of the cells was carried out according to Graham[19].

In the studies of oncogenicity 3-5 μg of the DNA (0.03-0.05 ml) were injected subcutaneously into the dorsal neck region of one-day old hamsters.

RESULTS

Infectivity of the SA7 DNA and the DNA - TP complex.

Table 1 presents typical experimental results obtained by titration of various preparations of the intact and cleaved SA7 DNA and the DNA - TP complex on monkey kidney cells untreated and treated with dimethylsulphoxide (DMSO). In the experiments without DMSO treatment the infectivity of deproteinized DNA and DNA - TP complex was found only at the concentration of 0.5-1 μg per 1 dish and the DMSO treatment sharply increased the infectivity. No differences were found between the infectivity titers of the DNA and DNA - TP complex after titration on the cells which had not been treated with DMSO. Considerable differences between the DNA and DNA - TP complex preparations were found only for titration on the cells pretreated with DMSO when the infectivity titers of the DNA - TP complex were 30-50 times higher than

Table 1. Infectivity of the SA7 DNA and DNA - protein complex

Preparation	DNA µg/dish	DMSO pre-treatment*)	PFU per dish**)		Mean PFU per 1 µg DNA per dish
1. DNA	1	-	6	3	4.5
2. DNA-protein complex	1	-	4	2	3.0
3. DNA-protein complex	0.5	-	2	0	2.0
4. DNA	1	+	19	130	74.5
5. DNA	0.1	+	2	7	45.0
6. DNA-protein complex	1	+	400	33	216.5
7. DNA-protein complex	0.1	+	93	116	1045
8. DNA-protein complex + pronase	1.0	+	3	5	4.0
9. DNA-protein complex + R.EcoRI	0.1	+	0	0	0.0
10. DNA-protein complex + DNAase	1.0	+	0	0	0.0

*) 10% DMSO for 6 min prior to infection

**) In each experiment titration was carried out simultaneously for 2 dishes

those for the deproteinized preparations. It was the TP linked to DNA which produced the difference between the titers since the pronase treatment of the complex decreased its infectivity to the level typical for the free DNA. A short time digestion of DNA preparations by DNAase and cleavage by R.EcoRI completely eliminated infectivity. Similar results were obtained in other experimental series though the infectivity levels of the DNAs and DNA - TP complexes varied.

Transformations of cells by the SA7 DNA and DNA - TP complex. As reported earlier[4], the transfection of kidney cells of newborn rats or rat embryo fibroblasts with the

SA7 DNA produced foci of transformation in 3-5 weeks after transfection. The efficiency of transformation of both cultures was the same (the data are not given) amounting to about 1 focus per 10^5 cells per 1 μg of the DNA (Table 2). Under the conditions used the transforming activity of DNA was on the average 0.53 focus per dish per 1 μg of the DNA. The DNA - TP complex did not transform the rat cells even after transfection of 2 μg of DNA per dish or after cleavage of the DNA - TP complex by R.BamHI (see below).

It should be noted here that the lack of transforming activity found in the experiments shows, strictly speaking, only the lack of activity over a certain level determined by the sensitivity of the detection technique. Proceeding from the data of Table 2 one may assume that the transfor-

Table 2. Transforming activity of the SA7 DNA and DNA-protein complex

Preparation	DNA μg/dish	No. of dishes		No. of foci	
		Total	With transformation foci	Total	Per 1 dish per 1 μg DNA
1. DNA	1	56	19	30	0.53
2. DNA-protein complex	1.2-2.0	16	0	0	0.00
3. DNA-protein complex + pronase	1.2	7	3	4	0.50
4. DNA+R.BamHI*)	1.5	20	6	9	0.30
5. DNA+R.SalI*)	1	19	11	22	1.2
6. DNA+R.EcoRI*)	2	16	4	5	0.15
7. DNA+DNAase	2	4	0	0	0.00
8. DNA-protein complex + R.BamHI	1	4	0	0	0.00
9. Chicken erythrocyte DNA	10	4	0	0	0.00

*) The mixture of DNA fragments after cleavage with the respective restrictase

ming activities of the DNA and the DNA - TP complex are identical and amount to 0.53 focus per dish per 1 μg of the DNA. Since the amount of DNA used in the experiments with the DNA - TP complex was 23 μg we should expect 12 transformation foci according to the above assumption. As no transformation foci were found for the DNA - TP complex it can be suggested that if the DNA - TP complex has any transforming potential at all it is weaker by a factor of 15 than that of the free DNA. The decrease or the lack of transforming activity of the complex can be attributed exclusively to the TP since when it is removed by pronase the transforming activity of DNA is restored to the normal level.

In contrast to the infectivity the transforming activity of the SA7 DNA was not eliminated by treatment with the restriction endonucleases (Table 2).

Actually, a three-fold decrease of the transforming activity with respect to the intact DNA was found only after cleavage of the SA7 DNA by R.EcoRI while cleavage by R.BamHI resulted in a very small change in the transforming activity, and cleavage with R.SalI even markedly enhanced the transforming activity. In fact, our results (see Discussion) should be regarded as indicating an increase in the transforming potential of DNA after cleavage with restrictases. In view of these results the lack of transformation of cells observed in the experiments with the DNA - - TP complex cleaved by R.BamHI suggests that both the intact complex and its fragments do not possess the transforming capacity. The lack of the transforming activity found for the mixture of the R.BamHI fragments produced from the DNA - TP complex shows that the ring form of the DNA - TP complex cannot account for the loss of the transforming activity.

As should be expected, digestion with the DNAase completely prevented transformation; DNA of the chicken erythrocytes did not stimulate transformation of cells even when its amounts were 5-10 times those of the SA7 DNA.

Tumorigenic activity of the SA7 DNA and DNA - TP complex. The data of Table 3 show a high tumorigenic activity

Table 3. Tumorigenic activity of the SA7 DNA and DNA-protein complex

Preparation	DNA dose per hamster, μg	Frequency of tumour induction	
		No. of tumours no. of animals**)	Induction efficiency
Intact DNA	3-5	6/34	0.17
Intact DNA-protein complex	3-5	1/22	0.04
Fragment mixtures:			
R.EcoRI	3	3/20	0.15
R.BamHI	3	5/13	0.38
R.SalI	3	5/16	0.31
Individual R.SalI fragments*) :			
A	1.1	-	-
B	0.7	0/5	-
C	0.6	7/11	0.64
D	0.3	0/5	-
E + F	0.2	0/5	-

*) The amounts of the individual DNA fragments were calculated from their weight ratio to the intact SA7 DNA[5] (A-37%, B-25%, C-20%, D-10%, and E+F-6%)

**) Number of survived animal is indicated

of SA7 DNA. Tumours were developed 49-55 days after subcutaneous injection of 3-5 μg of DNA to the newborn hamsters. Morphologically, the tumours were nondifferentiated sarcomas[4]. The tumorigenic activity of the DNA - TP complex was considerably lower than that of the deproteinized DNA and even this residual activity is, most probably, due to the presence of 5-20% of the free DNA in the preparations of the DNA - TP complex (see Discussion). Cleavage of DNA with R.EcoRI and R.BamHI resulted only in a slight decrease of the activity while cleavage with R.SalI noticeably enhanced the tumorigenic effect of the DNA. The largest increase of the tumorigenic activity calculated for the equivalent amount of the DNA was found for the individual fragment C

cleaved with R.SalI from the left side of the SA7 (Fig.1). Other R.SalI fragments did not induce tumour development. Hence, it can be suggested that the onc-gene of SA7 is localized in the left terminal fragment C cleaved from the SA7 DNA with R.SalI whose molecular weight[5] is 4.3×10^6 D. This weight is comparable with the results obtained for the DNAs of the human adenoviruses[20].

DISCUSSION

In our opinion, special attention should be paid to the increase in the transforming and tumorigenic activity of the SA7 DNA following its cleavage with restrictases which has been found in this study. It would be reasonable to assume that any in vitro treatment of DNA should result in a damage of the genetic code and a nonspecific decrease of the biological activity. Such damage can be caused by nuclease admixtures, hydrodynamic effects, oxidation and other factors. Thus, even if a restrictase cleaves the DNA without direct damage to the onc-gene the resulting mixture of the DNA fragments should have a lower transforming or tumorigenic activity, the other conditions being equal. It is just this factor that causes the decrease of the both types of activity when R.EcoRI acts on the only recognition site in the SA7 DNA breaking it into two fragments with the molecular weights 12 and 10×10^6 D respectively[3,5]. The same nonspecific damage of the DNA molecule should occur when other

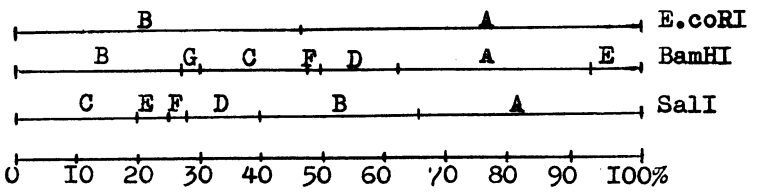

Fig.1. Physical map of the SA7 genome obtained by clevage with the restrictases R.EcoRI, R.BamHI and R.SalI. The fragments are presented as percentages of the genome size

restrictases act on the DNA but we failed to record it. Moreover, the activity of the mixture of 7 R.BamHI fragments was close to the activity of the starting DNA while the activity of the mixture of 6 R.SalI fragments was markedly higher (see the restriction map in Fig.1).

The observed paradoxical increase of the transforming and tumorigenic activity of the SA7 DNA with increasing degree of its fragmentation can be explained only if we assume that the activity of the fragment containing the onc-gene increases up to a certain level with the decrease in the fragment size. In this connection we should analyze the differences in the sizes of the left terminal DNA fragment with the onc-gene cleaved with different restrictases. As can be seen from Fig.1 the left fragments cleaved with R.EcoRI, R.BamHI and R.SalI have the molecular weights 10×10^6, 5.9×10^6 and 4.3×10^6 D respectively[5]. Thus, the transforming and oncogenic potentials of these fragments increase while their sizes decrease from 45% to 19% of the genome size. At present we are studying smaller fragments containing the onc-gene.

The observed increase of activity with decreasing size of the fragment containing onc-gene can be explained by analogy with bacterial transformation[21]. For genetic transformation of bacteria involving the general recombination system an optimum relationship has been demonstrated between the size of transforming DNA and the frequency of transformation. A similar effect has been found in the studies of DNA integration in the eukaryotic cells[22].

If we assume that a similar relationship is valid also for integration of the DNA of oncogenic viruses in transformation of eukaryotic cells we can conclude that the shorter (to a certain size) DNA fragments can have a higher transforming activity. Highly relevant results in this respect were reported by Doerfler[22] and other authors[23] who found that the viral sequences in the DNAs of the transformed cells were typically represented by relatively small parts of original genomes with the molecular weights from 2×10^6 to 5×10^6 D.

The above discussion raises the question of the "reference point" in determination of the stimulating effect of cleavage on the transforming and oncogenic activity of the SA7 DNA. The experimentally observed activity is determined by the effects of the two opposite factors (the nonspecific damage and the specific stimulation) and therefore we should take as the reference not the intact DNA but the mixture of R.EcoRI fragments which have the lowest biological activity. It is clear that the DNA fragment B containing the onc-gene is in this case large enough to preclude any advantages in integration in comparison with the intact DNA while allowing the highest possible damaging nonspecific effect of R.EcoRI cleavage. If the transforming activity of the mixture of the R.EcoRI fragments is taken for activity unit the relative activities of the mixture of R.BamHI fragments, the intact SA7 DNA and the mixture of R.SalI fragments are 2.0, 3.5 and 8.0, respectively (Table 2). Furthermore, if the tumorigenic activity of the mixture of R.EcoRI fragments is taken for activity unit the activities of the intact DNA, the mixture of R.BamHI fragments, the mixture of R.SalI fragments and the individual R.SalI fragment C are 1.1, 2.5, 2.1 and 6.4, respectively (Table 3). Thus, when the molecular weight of DNA fragment containing the onc-gene decreases from 10×10^6 to 4×10^6 D the neoplastic potential of the DNA increases 6–8 times.

The second noteworthy result of these studies is the observation that the DNA - TP complex either has a very low transforming and oncogenic activity or lacks it altogether while the infectious titers of the complex are 30–50 times higher than those for the deproteinized DNA.

In the analysis of the above results, particularly, in the evaluation of the fact that the DNA - TP complex has induced a tumour in a single experimental hamster (see Table 3), it should be born in mind that not all the DNA molecules in the complex are linked to the TP. There are no direct and exact data on the amount of the free DNA molecules but it can be indirectly estimated as 5–10% in the best preparations and 20–25% in the worst ones (E.Z.Goldberg, unpub-

lished results). This estimate correlates with the fact that only a part of DNA molecules in the DNA - TP complex of SA7 and other adenoviruses have ring conformations[8,9]. Moreover, from 5 to 20% of the terminal DNA fragments cleaved by restrictases from different DNA - TP complexes entered the agarose gel while 80-95% of the fragments remained at the top of the gel owing to the presence of the TP[8]. The residual tumorigenic and transforming activity of the DNA - TP complex could be attributed to the admixture of the free DNA.

The inhibiting effect of the TP on the transforming and tumorigenic activity of the DNA - TP complex can be attributed to the following reasons:

1. TP inhibits in some way the penetration of DNA through the cytoplasmic or nuclear membrane.

2. TP decrease the number of "transformation centres" by facilitating the aggregation of DNA.

3. TP precludes the integration of adenovirus genome or its onc-gene into the cellular genome.

4. TP inhibits expression of the onc-gene or other terminal genes which are necessary for cell transformation or tumour induction.

The first suggestion seems to be unlikely since the DNA - TP complex penetrates the monkey kidney cells (see Table 1). Moreover, absorption of DNA by the cell is based on active transport of the DNA[24] and a small amount of protein (0.5% of the DNA weight) linked to the DNA can hardly effect functioning of the permease system of the cell. Direct experiments with ^{32}P-labelled DNA and DNA - TP complex have shown that both these types of molecules penetrate the rat embryo kidney cells with the same rate and efficiency (E.Z.Goldberg, unpublished results). There is a paucity of data on the mechanism of DNA penetration through the nuclear membrane and this explanation should be specially analyzed.

The aggregation hypothesis also seems to be unlikely since our procedure for infection of cell with the DNA in the presence of Ca^{++} by itself produced considerable aggregation of the polynucleotide material[19]. Therefore, it can

be assumed that the numbers of the "transformation" centres in experiments with DNA and DNA - TP complex can hardly differ considerably. In some experiments we increased the amount of transfected DNA - TP complex 5-8 times but the results remained negative.

Covalent blocking of the 5' - ends of the polynucleotide chain in the DNA - TP complex, indeed, could preclude integration of the adenovirus and cellular genomes. Although there appeared some doubts concerning the covalent nature of the linkage of TP to 5' - ends in adenoviral DNA[25] we still consider this explanation the most plausible. Since infection with intact adenovirions also involves penetration of the DNA - TP complex, rather than free DNA into the cell, the infected cell should have a mechanism for reversible deblocking of the 5' - ends of the polynucleotide chain which, however, does not work in transfection of the rat cells with the DNA - TP complex.

Blocking of transcription of the onc-gene owing to the TP is close to the integration inhibition effect discussed above including the required deblockingof the 5' - end in the process of normal adenovirus infection. In this connection it would be worthwhile to establish whether terminal protein acts only when it is linked to DNA (cis-function) or it can manifest a trans-function preventing the transforming or tumorigenic effect of the free DNA if they are simultaneously introduced into the cell.

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