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Studies of the transcription of viral genome in adenovirus 5 transformed cells

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ABSTRACT

Transcription of the human adenovirus 5 genome in transformed rat embryo cells (DFK3) was investigated using two different approaches. Preferential digestion of transcribed viral sequences by DNase I was analysed using kinetics of renaturation of ^{32}P-labeled Ad5 HpaI restriction fragments in the presence of material which was stable after nuclease treatment. The second approach was the hybridization of ^{32}P-labeled nuclear RNA from transformed cells with Ad5 restriction fragments which were attached to a nitrocellulose filter. These two methods gave similar results. It was found that not all integrated regions of the Ad5 genome are active in transformed cells.

2,5 copies of the HpaI-E fragment of Ad5 DNA were found in transformed DFK3 cell line. Nuclear RNA from these cells hybridized to HpaI-E fragment of Ad5 DNA, but only about half of sequences of the integrated HpaI-E fragment was sensitive to DNase I digestion.

INTRODUCTION

Studies of the transcription of adenovirus genes in transformed cells are of importance in order to elucidate the regulation of gene expression in eukaryotic cells as well as the mechanism of cell transformation.

So far, the only method which permits one to investigate transcription of viral genes is the analysis of RNA-transcripts /1-5/. Using the technique of reassociation kinetics with specific fragments of viral DNA as probes, it has been shown that the extent of viral sequences and the number of copies present per diploid quantity of DNA extracted from cells transformed by either adenovirus type 2 or type 5, varies in different cell lines/6,7/. Hybridization of RNA from the transformed cells to restriction endonuclease generated fragments of viral DNA has shown that only a portion of

the viral sequences are transcribed /5/. From this type of analysis it is not clear whether all of the copies of a certain sequence are active in transcription, however, with a few exceptions, the number of RNA transcripts of a given sequence closely parallels the number of copies of viral DNA, from cell line to cell line /8/.

A method which permits examination of transcription in a way such that one may be able to determine how many copies of a particular gene are transcriptionally active is based on the observation that after digestion of nuclei or chromatin by DNase I, sequences that are active in transcription are selectively digested /9/.

The purpose of the present work was to determine the pattern of transcription of Ad5 DNA in transformed cells. Two different methods were employed: hybridization of in vivo labeled ^{32}P RNA with restriction fragments of viral DNA and reassociation of fragments of viral DNA in the presence of transformed cell DNA which had been selectively digested with DNase I.

MATERIALS AND METHODS

Cells. Rat embryo cells transformed by Ad5, line DFK3, were grown in glass roller bottles (New Brunswik) in Eagle's minimal essential medium (MEM), supplemented with 10% calf serum. KB cells for cultivation of Ad5 virus were grown in suspension culture in spinner modified MEM supplemented with 5% calf serum.

^{32}P labeled cells were prepared in the following manner. Cells were washed with the phosphate free MEM, containing 25 mM HEPES and 1% dialysed calf serum and then incubated for 4 hr in the same medium with 500 µCi of $H_3{}^{32}PO_4$ (carrier free Amersham) per ml.

Virus. Ad5 was propagated in suspension culture of KB cells at an input multiplicity of 10 PFU/cell. Virus was purified by a modification of the method of Green and Piña /9,10/.

Viral DNA. Ad5 DNA was extracted from purified virions as described /11/.

Cell DNA. Nuclei from transformed cells were isolated, digested with DNase I (Worthington) as described /9/ and DNA from native and DNase I treated nuclei was isolated /9/.

RNA. ^{32}P-labeled RNA was extracted from isolated nuclei of DFK3 cells and purified by the method of Scherrer /12/.

Restriction endonucleases. Endonuclease EcoRI was isolated from E.coli strain RY-13 /13/. HpaI was preparated from H.parainfluenzae according to Sharp et al. /14/.

Preparation of specific fragments of adenovirus 5 DNA. HpaI restriction fragments of Ad5 DNA were separated by electrophoresis on 0,7x30 cm cylindrical gels of 0,7% agarose /5/ and prepared as described by Gallimore et al. /15/. HpaI+ EcoRI restriction fragments of Ad5 DNA were separated by electrophoresis on a 0,4x12x15 cm slab gel of 1% agarose.

Radioactive labeling of DNA. HpaI restriction fragments of Ad5 DNA were labeled to specific activities of 1-8x10^{7}cpm/µg using four /α-^{32}P/deoxynucleotide triphosphates (250Ci/mmol, Amersham) and DNA-polymerase I (fractionVII, Boehringer Mannheim Biochemicals) according to Botchan et al. /16/.

Blotting. The fragments of Ad5 DNA were transferred from the gel onto a sheet of nitrocellulose (B6, Schleicher and Schüll) using essentially the method described by Southern/17/.

Hybridization conditions. DNA:RNA filter hybridization was carried out at 65^{o}C for 16 hr in 6xSSC, 0.5% SDS, 0.001 M EDTA. Before hybridization a strip of nitrocellulose filter (0.3x11cm) was soaked in a solution containing 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone dissolved in 2xSSC /18/ for a period of 4-5 hr at 65^{o}C. The filter was then incubated under the above conditions with 10x10^6 cpm of ^{32}P-labeled nuclear RNA from DFK3 cells in a volume 0.2 ml. Following hybridization the nitrocellulose strip was washed with 2xSSC, incubated in 7 M urea, 2xSSC, 0.5% SDS at 42^{o}C for a period of 2 hr, again washed with 2xSSC, air-dried and subjected to autoradiography (No-Screen Film, Type NS-5T, Kodak) for one week.

DNA:DNA hybridization was carried out at 68^{o}C in 1.0 M NaCl buffered with 0.14 M Na-phosphate pH 6.8. Before annealing both viral and cellular DNA were degraded to oligo-

nucleotides with the average size ~150 bases by boiling in 0.3 M NaOH for 30 min. DNA from nuclei digested with DNase I had the same average length, as judged by sedimentation in the alkaline sucrose gradient (data not shown). Samples taken after different times of incubation at 68°C were diluted 10-fold in 0.14 M Na-phosphate pH 6.8 and stored at 4°C until assayed by chromatography on hydroxylapatite /6,15/.

Calculations. The derivation of equation and methods of calculation have been described in detail elsewhere /6/.

The time required for 50% of the ^{32}P-labeled probe to reanneal in the presence of calf thymus DNA ($t_{1/2}p$) and in the presence of transformed cell DNA ($t_{1/2}$) was calculated from equation (1):

$$t_{1/2}(\text{or } t_{1/2}p) = \frac{1}{1/fss - 1} \qquad (1)$$

where fss is the fraction of total ^{32}P-labeled DNA that is single stranded at time t, the time of reannealing.

The quantity of viral DNA per diploid quantity of transformed cell DNA (3.9×10^{12} Daltons) /19/ was calculated as described by Sharp et al. /6/.

RESULTS

Before using specific fragments of Ad5 DNA generated by cleavage with restriction endonuclease HpaI (Fig.1) in an analysis of the viral DNA sequences present in different DNA preparations from DFK3 cells, it was necessary to demonstrate that all fragments were capable of annealing to viral DNA sequences. The conditions in which ^{32}P-labeled probe DNA was annealed in the presence of transformed cell DNA were closely reproduced in reconstruction experiment in which the hybridization mixtures contained a large amount of unrelated, control cell DNA and a small amount of viral DNA. The data obtained were treated as described in Materials and Methods to give the values shown in part 1 of Table I. The rates of reannealing of all ^{32}P-labeled fragments were accelerated in the presence of unlabeled Ad5 DNA by an amount close to that expected from the quantity of viral DNA added. Thus we conclude that the HpaI fragments of Ad5 DNA reanneal at rates proportional to

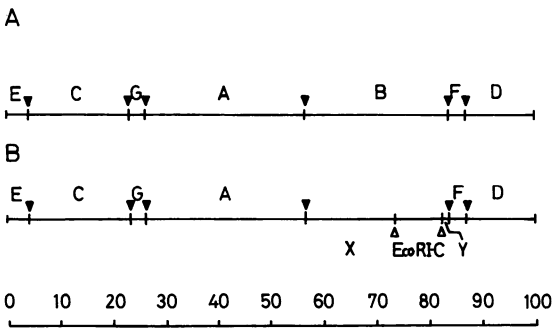

Fig.1. A. Hemophylus parainfluenzae restriction endonuclease (HpaI) cleavage map of the adenovirus 5 genome /5/. B. HpaI+EcoRI cleavage map of the adenovirus 5 genome /5/. ▼ - HpaI cuts; EcoRI cuts - ∇ .

Table I. Renaturation of ^{32}P-labeled fragments of Ad5 DNA in the presence of DNAs extracted from native and digested with DNase I nuclei of DFK3 cells.

DNA preparation	Fragment		$(t_{1/2}p/t_{1/2})$	Equiv./diploid quant. cell DNA, assuming sequences homologous to complete fragment
1. 1.77×10^{-2}ug/ml Ad5 DNA and 1.0mg/ml calf thymus DNA	HpaI	A+B	16.02±0.24	2.9
		C	6.18±0.43	2.8
		D	3.95±0.15	3.1
		E	2.13±0.24	3.3
		F	2.31±0.11	3.5
		G	1.69±0.25	3.3
2. 1.0mg/ml DNA from nuclei of DFK3 cells untreated with DNase I	HpaI	A+B	3.87±0.35	0.6
		C	3.02±0.11	1.1
		D	2.53±0.64	1.6
		E	1.96±0.21	2.8
		F	2.36±0.81	3.3
		G	1.21±0.34	1.1
3. 1.0mg/ml DNA from nuclei of DFK3 cells treated with DNase I	HpaI	A+B	3.14±0.15	0.4
		C	1.40±0.23	0.2
		D	1.14±0.15	0.0
		E	1.44±0.15	1.3
		F	1.10±0.01	0.0
		G	0.85±0.03	0.0

their initial molar concentrations in the reaction mixture. The effect of transformed cell DNA on the reassociation kinetics of the ^{32}P-labeled fragments can be used to determine the concentration of each fragment in different DNA preparations from DFK3 cells.

Digestion of viral DNA sequences in DFK3 cells with DNase I. The rates of reannealing of ^{32}P-labeled HpaI fragments of Ad5 DNA were measured in the presence of unlabeled

DNA extracted from native nuclei of DFK3 cells, nuclei which were digested with DNase I and calf thymus DNA. The results of renaturation experiments are shown in Figure 2 and Table I. Such assay permits the detection of a preferential sensitivity or resistance of specific portions of the integrated adenovirus DNA to DNase I.

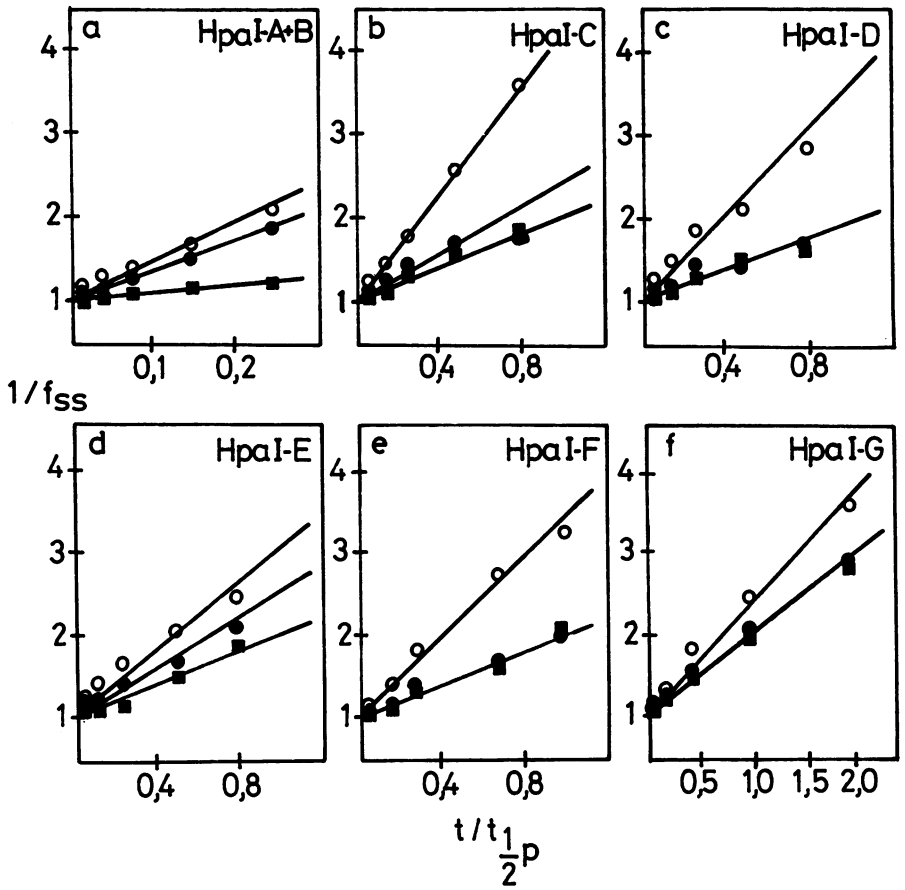

Fig.2. Kinetics of reassociation of ^{32}P-labeled HpaI fragments of Ad5 DNA in the presence of calf thymus DNA (■) and DNA extracted from the native nuclei of DFK3 cells (o) and nuclei of DFK3 cells digested with DNase I (●). The concentration of calf thymus DNA and DNAs extracted from DFK3 digested and undigested nuclei was 1.0 mg/ml. The concentrations of the ^{32}P-labeled probe DNAs were HpaI fragments A+B, 6.8×10^{-4} µg/ml; HpaI fragment C, 6.39×10^{-4} µg/ml; HpaI fragment D, 5.99×10^{-4} µg/ml; HpaI fragment E, 7.05×10^{-4} µg/ml; HpaI fragment G, 6.40×10^{-4} µg/ml.

DNA extracted from untreated nuclei of DFK3 cells increased the rate of renaturation of all HpaI restriction fragments of Ad5 DNA. The numbers of copies of different fragments per diploid quantity of DFK3 DNA (part 2 of Table I) were in accord with the data obtained earlier /20/.

DNA isolated from DNase I digested nuclei of DFK3 cells (nuclei were digested with DNase I until 25% of DNA became acidsoluble) increased the rate of reassociation only of ^{32}P-labeled E and A+B fragments. Other fragments reassociate with the same rates as in the presence of calf thymus DNA (Fig 2 a-f).

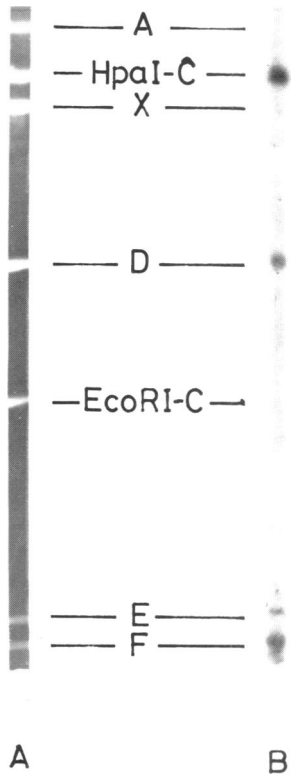

Fig.3. Hybridization of ^{32}P-labeled nuclear RNA extracted from DFK3 cells with HpaI+EcoRI restriction fragments of Ad5 DNA. HpaI+EcoRI restriction fragments of Ad5 DNA after electrophoresis in 1% agarose gel (A). Autoradiograph of Ad5 restriction fragments after hybridization with nuclear ^{32}P-labeled RNA of DFK3 cells (B). Fragments Y and HpaI-G have run off the gel.

The data indicates that the preferential complete elimination of HpaI fragments C,D,F and G occured during DNase I digestion of nuclei of DFK3 cells. Sequences which are complementary to HpaI fragments E and A+B are present in DNA from DNase I digested nuclei in distinguishable amounts (Fig.2a,d). The number of copies of A+B and E fragments which remain stable after DNase I digestion of nuclei was calculated (part 3 of Table I).

Viral RNA sequences in DFK3 cells. Viral nuclear RNA sequences in this cell line were assayed on nitrocellulose filter by hybridization of ^{32}P-labeled RNA extracted from the nuclei of transformed cells to specific endonuclease restriction fragments of unlabeled Ad5 DNA. For hybridization experiments we have used specific fragments which were obtained after simultaneous digestion of unlabeled Ad5 DNA with HpaI and EcoRI restriction endonucleases. After treatment of Ad5 DNA with these two enzymes we were able to separate in 1% agarose slab gels HpaI fragment A and two main fragments which EcoRI produced from HpaI fragment B (X and EcoRI-C).

The results of hybridization are shown in Figure 3. RNA extracted from the nuclei of DFK3 cells hybridized only to HpaI fragments C, D, E and F. No radioactive spots were observed at the positions of HpaI-A and HpaI-B (X and EcoRI-C) Ad5 restriction fragments.

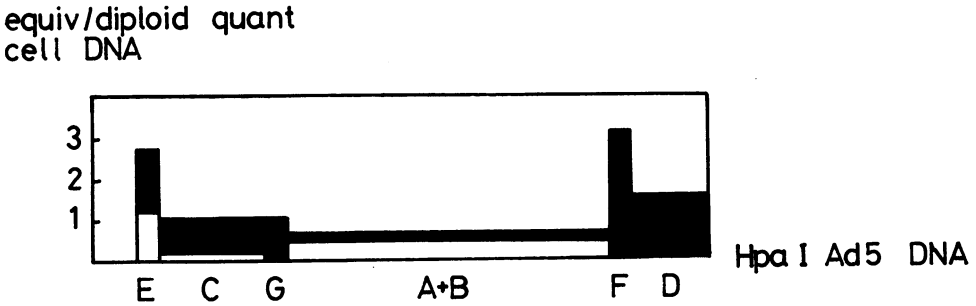

Fig.4. Pattern of transcription of the integrated adenovirus 5 genome in DFK3 cell line.
■ - transcribed sequences; □ - nontranscribed sequences.

Thus, DFK3 transformed cells seem to express into nuclear RNA those sequences of the Ad5 genome which are complementary to C, D, E and F HpaI fragments of Ad5 DNA. Although some sequences of HpaI fragments A+B are present in DFK3 cells (0.6 copies per diploid quantity of DFK3 DNA) (part 2 of Table I, and ref.20) RNA-transcripts which are complementary to HpaI fragments A and B (X and EcoRI-C) are absent in the nuclei of DFK3 cells, or their amount is less than 10^{-5} of nuclear RNA (we were able to detect 100 cpm per spot).

The obtained data indicate that not all sequences of Ad5 DNA which are present in DFK3 cells are transcribed.

DISCUSSION

The DFK3 transformed cell line was obtained after treatment of rat embryo cells by Ad5 virus. The fragments of the integrated viral genome in this cell line are present in different numbers of copies /20/. The same results were obtained with different Ad2 and Ad5 transformed cell lines /5,7,15,20/. The only segment of viral DNA which was common for all of these lines was the 14% of the extreme left-hand end of the viral genome. Graham et al. have found that 7% of the left-hand end of the viral genome is sufficient for transformation /21/. So any viral functions required for the maintenance of the transformed cell phenotype are encoded by about 7-8% of the Ad5 genome and map at the extreme left end.

The HpaI-E fragment of the Ad5 genome is present in 2.5 copies per diploid quantity of cell DNA in the DFK3 line. The hybridization of nuclear RNA from this cell line to separated restriction fragments of Ad5 DNA shows that transcription of the E fragment and also C, D and F occurs in transformed cells. But the problem with all hybridization experiments detecting the transcription of some viral sequences in transformed cells is the difficulty in distinguishing between functional and randomly integrated viral DNAs. The only method which permits one to overcome this problem is the method of preferential digestion of transcribed DNA sequences in nuclei with DNase I and the following analysis of stable material. This method was developed by Weintraub and Groudine for analysis of hemoglobin genes /9/.

The analysis of the expressed Ad5 sequences in DFK3 transformed cells by this technique indicates that the method is sensitive enough for analysing of transcription of viral genomes in transformed cells (Fig.2,3) and that not only the extreme left end of the genome is transcribed, but HpaI fragments from the majority of the genome are transcribed as judged by DNase I sensitivity (Fig.2). This observation is in agreement with the results of other authors /1,2,4/.

The obtained data show that about 50% of the sequences of fragment HpaI-E are sensitive to DNase I digestion of DFK3 cell nuclei. Simple explanations of this fact are that not all identical sequences of this fragment are active in transcription or/and 50% of the length of HpaI-E fragment are transcribed and sensitive to DNase I digestion of nuclei (Fig.4).

The precise explanation may be obtained in the experiments with restriction fragments of HpaI-E and after investigation of pattern of the integrated viral sequences in DFK3 cells.

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