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The release of growth arrest by microinjection of adenovirus E1A DNA

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The induction of DNA synthesis in growth-arrested mouse fibroblasts (NIH 3T3) was studied by microinjection of different constructs of adenovirus DNA using SV40 DNA and plasmid DNA as positive and negative controls. The E1A region of adenovirus types 2 and 12 appears to be sufficient to induce cellular DNA synthesis after growth arrest in ~30% of the cells and both 13S and 12S cDNA constructs mediate this effect. The presence of the E1A protein products as assayed by immunofluorescence does not strictly correlate with the induction of DNA synthesis in microinjected cells in contrast to the SV40 large T-antigen. Microinjection of truncated fragments of the Ad12 E1A region suggests, however, that the protein products of 12S and 13S may be involved in the induction process. A sequence comparison of the SV40 T-antigen and the adenovirus E1A products identified a region of significant homology providing a basis for a hypothesis concerning the evolution of T-antigen genes in DNA viruses.

Key words: growth arrest/microinjection/DNA synthesis/sequence homology

Introduction

Infection of quiescent rodent cells with human adenoviruses induces the cells to progress from the G_0- or G_1-phase into the S-phase of the cell cycle and to synthesize cellular DNA (Shimojo and Yamashita, 1968; Strohl, 1969; Zimmerman and Raska, 1972; Schnipper *et al.*, 1973; Younghusband *et al.*, 1979; Rossini *et al.*, 1979; Braithwaite *et al.*, 1981). The identification of the viral gene product(s) responsible for the induction of cellular DNA synthesis is of interest not only to understand virus-host cell interactions, but may also help to elucidate the regulation of the G_0-G_1-S-phase transition in the cell cycle.

Approaches to identify the DNA replication-inducing activity of the adenovirus genome first involved infection of quiescent cells with viral temperature-sensitive mutants or deletion mutants (Braithwaite *et al.*, 1981, 1983; Spindler *et al.*, 1985). These studies demonstrate that the early region E1A was the only viral function required for induction of host cell DNA synthesis in mouse cells arrested in G_0 by serum starvation (Murray *et al.*, 1982; Braithwaite *et al.*, 1983).

The E1A gene encodes three overlapping mRNAs that have identical 5′ and 3′ termini. They differ in the size of their introns. Each uses a common 3′-splice acceptor, but differs in the location of the 5′-splice donor, giving rise to mRNAs with sedimentation coefficients of 13S, 12S and 9S (cf. Figure 2).

In contrast to other early viral genes the E1A transcription unit is efficiently expressed after plasmid transfection (Svensson *et al.*, 1983; Weeks and Jones, 1983), facilitating the functional analysis of the E1A gene products.

Transfection of recombinant plasmids which specifically expressed the E1A 13S or the 12S mRNA, respectively (Svensson *et al.*, 1983) into HeLa cells demonstrated that only the 13S mRNA stimulates the expression of both viral and cellular genes by a trans-acting mechanism (Svensson and Akusjärvi, 1984). The E1A 12S mRNA had no detectable stimulatory effect on the expression of a plasmid-coded cellular β-globin gene and very little or no stimulatory effect on the expression of the adenovirus early region E4 (Winberg and Shenk, 1984; Svensson and Akusjärvi, 1984). The 12S mRNA product has, however, been reported to increase transcription from the viral promoters controlling early regions E2A and E3 (Leff *et al.*, 1984).

Studies to define the role of the individual E1A proteins in oncogenic transformation of non-permissive cells using viral mutants (Graham *et al.*, 1978; Ricciardi *et al.*, 1981; Carlock and Jones, 1981) and plasmid constructs (Bos *et al.*, 1983) provided evidence that *at least* the 289 amino acid protein encoded by the 13S mRNA is required for complete transformation. Transformation with E1A mutants expressing either the 289 amino acid protein or the 243 amino acid protein induced only a partially transformed phenotype (Montell *et al.*, 1984; Haley *et al.*, 1984; Hurwitz and Chinnadurai, 1985) indicating that both E1A proteins are required for complete transformation.

Expression of only the 12S mRNA coding for the 243 amino acid protein was reported to increase the transformation frequency even above the level seen with wild-type virus (Montell *et al.*, 1984; Haley *et al.*, 1984) and was found to be particularly important for anchorage-independent growth of the transformed cells (Montell *et al.*, 1984; Hurwitz and Chinnadurai, 1985).

The two major E1A products only differ by 46 amino acids which are unique to the 289 amino acid protein. Considering the apparent functional differences between these two closely related proteins with respect to activation of transcription of viral and cellular genes on the one hand, and transforming activity on the other, it is necessary to investigate the role of the individual products in the induction of cellular DNA synthesis.

The infection of quiescent mouse embryo cells with Ad5 deletion mutants demonstrated that E1A functions are required for induction of host cell DNA synthesis by the virus (Braithwaite *et al.*, 1983). Infection of serum starved rat cells with the Ad2 mutants dl 1500 and pm 975, which are able to express the 12S mRNA or the 13S mRNA, respectively, revealed that only the virus expressing the 12S mRNA product could stimulate cellular DNA synthesis, whereas the mutant pm975 expressing the 13S mRNA did not reproducibly enhance cellular DNA synthesis (Spindler *et al.*, 1985).

We have used a different approach to analyse the individual E1A products for their ability to trigger the transition from G_0- to S-phase in the cell cycle of a rodent cell. Plasmids containing modified E1A regions which have been shown to express either of the Ad2 E1A mRNAs (Svensson *et al.*, 1983; Montell *et al.*, 1982) were microinjected into quiescent mouse 3T3 fibroblasts and the cells were screened for induction of DNA synthesis by

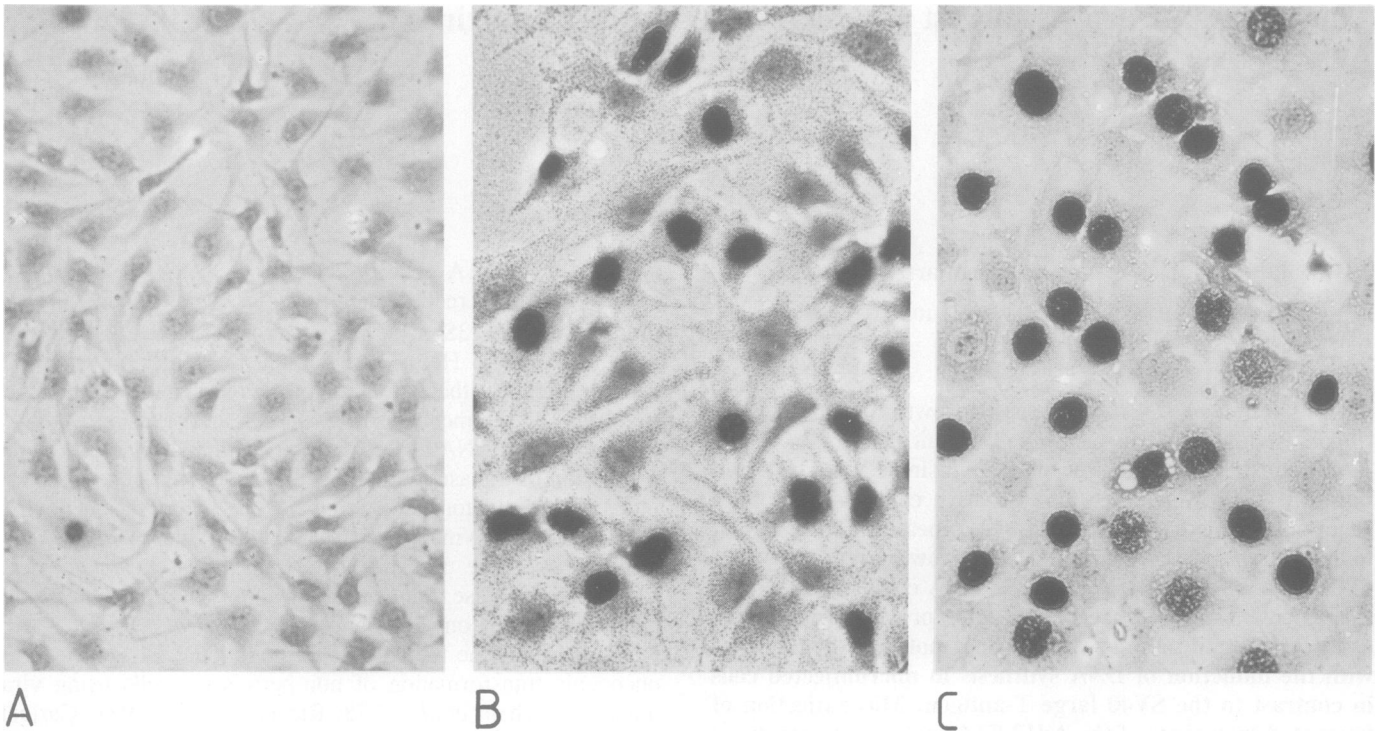

Fig. 1. (**A**) Plasmid pBR322 DNA (100 μg/ml), (**B**) Ad2 DNA (300 μg/ml) or (**C**) SV40 DNA (100 μg/ml) was injected into the nuclei of mouse NIH3T3 fibroblasts kept in 2.5% platelet-poor plasma for 48 h. After injection cells were labelled with [^{3}H]thymidine (1 μCi/ml) for 24 h. Cells were fixed, covered with autoradiographic stripping film Kodak AR10 and exposed for 2 days. The film was developed, cells were stained and photographed. Black nuclei show active DNA synthesis.

incorporation of [^{3}H]thymidine and autoradiography. Our results demonstrate that E1A-carrying plasmids expressing either 13S mRNA or 12S mRNA can induce DNA synthesis to a similar extent in this assay. Recombinant plasmids able to express only the 9S mRNA had no DNA synthesis-inducing activity.

Results

Microinjection of Ad2 DNA induces DNA synthesis in quiescent mouse 3T3 fibroblasts

Mouse NIH 3T3 fibroblasts are growth-arrested when the medium is deficient in platelet-derived growth factor (PDGF). Though platelet-poor plasma prepared from bovine blood contains low levels of PDGF, it possesses all other components required for growth (Vogel *et al.*, 1978).

Maintenance of NIH 3T3 fibroblasts for 3 days in medium containing 2.5% platelet-poor plasma resulted in growth arrest; within a subsequent 24-h labelling period, not more than 0.1 – 1% of the cells synthesized DNA as determined by incorporation of radioactive thymidine into the nucleus. Addition of 20 ng/ml of pure PDGF to the arrested cells induced DNA synthesis in >90% of the cells (data not shown).

The requirement of PDGF for induction of cellular DNA synthesis could be overcome by microinjection of Ad2 or SV40 DNA, but not plasmid DNA into the nucleus of quiescent cells (Figure 1). It has been shown that incorporation of radioactivity into the nucleus under similar conditions is due to replication of cellular DNA both after viral infection (Braithwaite *et al.*, 1981; Spindler *et al.*, 1985) or after microinjection (Floros *et al.*, 1981; Hyland *et al.*, 1984). Induction of DNA synthesis could never be observed in the present experiments after injection of plasmid DNA or bovine serum albumin (Table I) in contrast to previous results suggesting that microinjection of pBR322 DNA can stimulate DNA synthesis in quiescent Swiss 3T3 cells (Hyland *et al.*, 1984).

Induction of cellular DNA synthesis by microinjection of recombinant plasmids

To define the adenovirus region responsible for the induction of cellular DNA synthesis, recombinant plasmids carrying different constructs of adenovirus DNA were microinjected into the nuclei of growth-arrested mouse NIH 3T3 cells (Figure 2). Sets of 50 cells were injected per experiment, labelled for 24 h and screened for incorporation of radioactivity by autoradiography. Injection of intact Ad2 DNA triggers DNA synthesis in about 44% of the injected cells, a significantly smaller percentage than observed after injection of SV40 DNA which induced 73% of the cells (Table I). To understand the effect of prokaryotic sequences on the expression of viral proteins, we also injected SV40 DNA cloned in *Bam*I site of the plasmid pBR322 (pBR322-SV40) and tested the cells for expression of SV40 T-antigen and induction of cellular DNA synthesis. The ability of SV40 DNA to stimulate cellular DNA synthesis seems to be reduced by linking it to prokaryotic DNA sequences. The percentage of injected cells responding drops from 73 to 52% (Table I).

Injection of plasmid pKGO 170 containing the leftmost 17% of the Ad2 genome representing the entire E1A and E1B region leads to the same response as intact Ad2 DNA, establishing that region E1 is sufficient to induce cellular DNA synthesis to the same extent as intact Ad2 DNA. Presence of the SV40 enhancer upstream of region E1 in pKGO 170-SV reduces the response, possibly reflecting the recently described repression that adeno E1A exerts on the SV40 enhancer (Velcich and Ziff, 1985).

Recombinant plasmids constructed to express either the 13S mRNA (pKGO 13S) or the 12S mRNA (pKGO 12S) induce progression from G_0- to S-phase in about 29 or 32% of the cells,

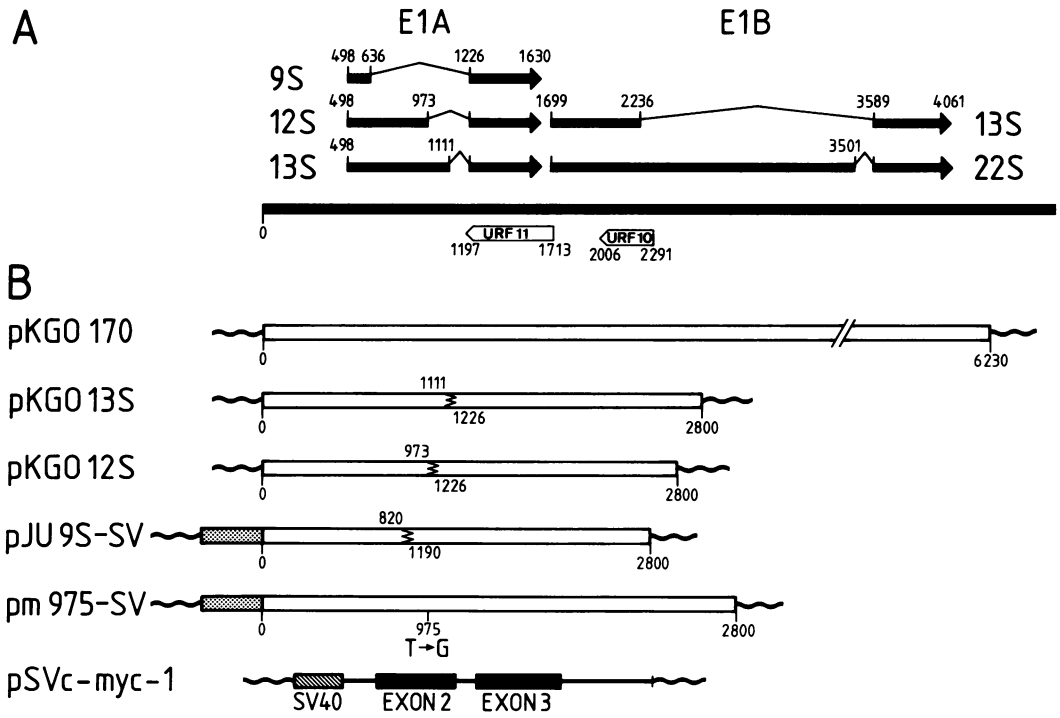

Fig. 2. Organization of the Ad2-early region E1 and recombinant plasmids. (**A**) Structure of mRNAs and unidentified reading frames (URF) encoded within regions E1. The location of capped nucleotides of the E1A and E1B mRNAs is from Baker and Ziff (1980, 1981), the location of the splice junctions for the E1A 12S and 13S mRNAs and the E1A poly(A) addition site are from Perricaudet *et al.* (1979). The position of the E1A 9S mRNA splice site is from Virtanen and Pettersson (1983) and the E1B cap and splice sites are from Perricaudet *et al.* (1980). Unidentified reading frames (URF) which have been detected in the l-strand sequence, are indicated (Sussenbach, 1984). (**B**) Schematic representation of recombinant plasmids used for microinjection. Construction of Ad2 deletion mutants was described by Svensson *et al.* (1983). Numbers indicate nucleotide positions from the molecular end of the Ad2 genome. Jagged line denotes deletion of nucleotides between the indicated positions. Stippled box represents the 366-bp *Hind*III-*Kpn*I fragment of SV40 DNA (nucleotide 5171-294), including the 72-bp repeat, the early promoter and the origin of replication (Svensson *et al.*, 1983). The pSVc-*myc*-1 clone was described by Land *et al.* (1983). It contains the 2nd and 3rd exon of the mouse c-*myc* gene coupled to the early SV40 promoter (striped box). Wavy line represents plasmid sequences.

respectively. Concurrent injection of the clones pKGO 12S and 13S did not give a higher percentage of induced cells (not shown). The SV40 enhancer seems again to reduce the response.

Splicing of the E1A mRNAs is non-sequential (Svensson *et al.*, 1983). Each of the three mRNA products is produced from an unspliced nuclear precursor and not from an intermediate, i.e., 13S mRNA does not mature into 12S or 9S mRNA. Although the construct pKGO 13S lacking the 13S mRNA intervening sequence still contains the splice donor site for 12S and 9S mRNA, it only expresses 13S mRNA (Svensson *et al.*, 1983). To confirm our results obtained with the clone pKGO 13S and to rule out that any 12S mRNA was generated from this construct we also tested a different construct pm975-SV. In this mutant a T-G transversion was introduced in the second position of the 12S intron by oligonucleotide mutagenesis (Montell *et al.*, 1982) thus preventing splicing of the 12S mRNA and allowing expression only of the 13S mRNA (Montell *et al.*, 1984). Injection of this clone gave a similar result as injection of pKGO 13S (Table I).

The construct pJU9S-SV carrying a deletion which removes the splice donor sites for both the 13S and 12S mRNA and thus only allows expression of the 9S mRNA failed to stimulate DNA synthesis. Injection of the prokaryotic vector DNA pBR322-SV still containing the SV40 enhancer did not induce cellular DNA synthesis.

Table I. Induction of DNA synthesis after microinjection of viral DNA and recombinant plasmids

DNA injected (100 μg/ml)	Number of experiments	Total number of cells injected	Labelled nuclei (%) ± standard deviation	Standard error of the mean
SV40	18	900	73 ± 13.9	3.3
pBR322-SV40	8	400	52 ± 6.7	2.4
Ad2 (300 μg/ml)	19	950	44 ± 14.4	3.3
pKGO170	15	750	57 ± 16.4	4.2
pKGO170-SV	12	600	34 ± 9.0	2.6
pKGO13S	14	700	29 ± 13.6	3.6
pm975-SV	18	900	24 ± 7.0	1.7
pKGO 12S	18	900	32 ± 9.7	2.3
pKGO 12S-SV	14	700	28 ± 9.3	2.5
pJU 9S-SV	10	500	1 ± 1	0.3
pBR322-SV	4	200	0 ± 0	
pSVc-myc-1	8	400	4 ± 3.4	1.2
pSV2-β globin	2	100	1 ± 1	0.7
Bovine serum albumin	4	200	1 ± 1	0.5

Viral DNA or recombinant DNA plasmids as detailed in Figure 2B were injected into 50 cells per experiment. Cells were labelled for 24 h with [^{3}H]thymidine and exposed for autoradiography. For each experiment the proportion of injected cells responding with DNA synthesis was determined. SV denotes presence of the SV40 enhancer element (cf. Figure 2).

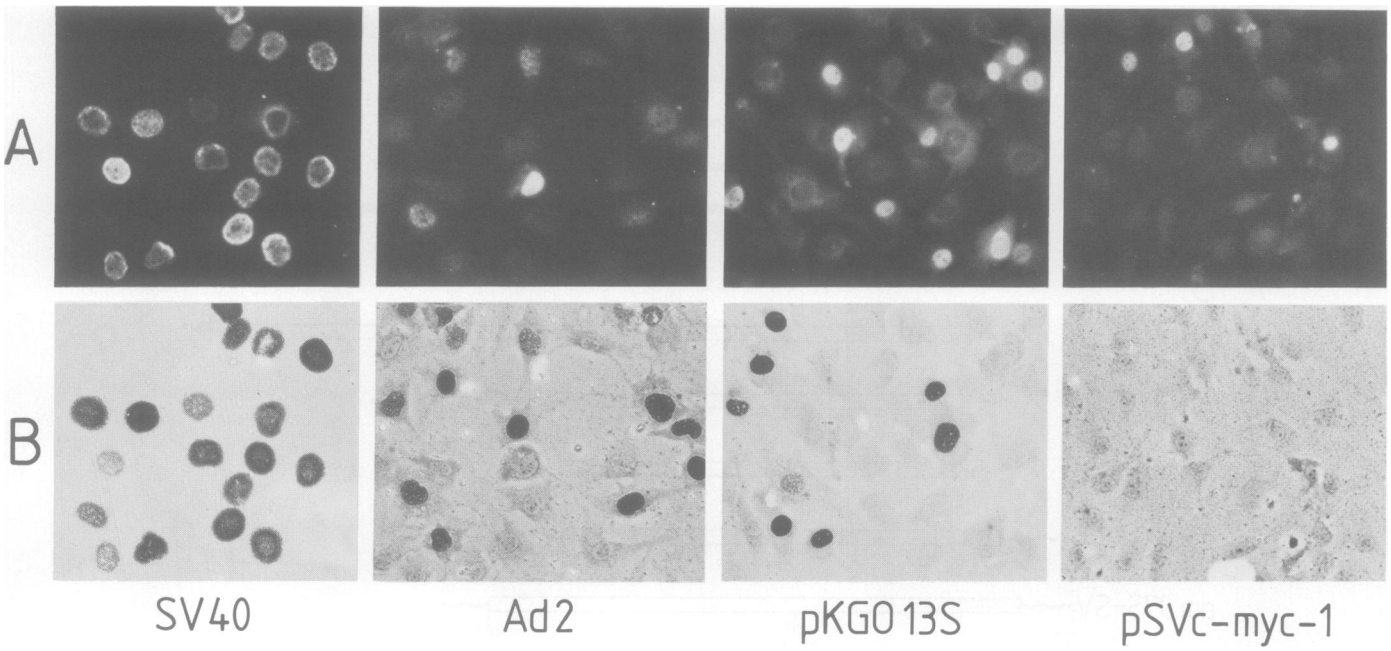

Fig. 3. Correlation between expression of injected genes and induction of cellular DNA synthesis. Cells were injected with SV40 DNA (100 μg/ml), Ad2 DNA (300 μg/ml), plasmid DNA pkG013 S (1 mg/ml) or plasmid pSVc-*myc*-1 DNA (1 mg/ml) as indicated and labelled with 1 μCi/ml [^{3}H]thymidine. At 24 h after injection, cells were fixed, processed for indirect immunofluorescence detecting SV40 T-antigen, E1A antigens or mouse *myc* protein, respectively, and photographed. Cells were then exposed to autoradiographic film and the pattern of radioactively labelled nuclei in the autoradiograms (**B**) was compared to the pattern of immunofluorescent nuclei (**A**).

The mouse cellular myc gene does not induce DNA synthesis

Studies on the cooperativity of oncogenes have indicated that similar features exist in the adenovirus early region E1A and the cellular oncogene *myc* with regard to their ability to 'immortalize' primary rodent cells and render them susceptible for malignant transformation by a second oncogene like Ha-ras, N-ras or polyoma middle-T (Land *et al.*, 1983; Ruley, 1983). We therefore investigated whether *myc* could also induce cellular DNA synthesis.

Quiescent cells were injected with a plasmid expressing the second and the third exon of the mouse cellular *myc* gene from the SV40 early promoter as described by Land *et al.* (1983) (Figure 2).

Expression of the *myc* protein in the injected cells was confirmed by indirect immunofluorescence using a polyclonal antiserum (Figure 3). Only a few cells injected with the pSVc-*myc*-1 construct and expressing the protein were induced to enter the S-phase (Table I) indicating that enhanced expression of the cellular *myc* gene is not sufficient to stimulate cellular DNA synthesis in this system.

Correlation between expression of the E1A proteins and induction of DNA synthesis

Induction of cellular DNA synthesis by SV40 DNA is mediated by large T-antigen. All cells which were stimulated to synthesize DNA after infection with SV40 or microinjection of SV40 DNA also expressed T-antigen at the same time (Tjian *et al.*, 1978; Mueller *et al.*, 1978). In accordance with the findings of Mueller *et al.* (1978) most if not all of the cells expressing T-antigen after injection of SV40 DNA or a plasmid construct containing SV40 DNA also had incorporated [^{3}H]thymidine into the nucleus (Figure 3).

The expression of E1A proteins after microinjection of intact Ad2 DNA or mutated E1A region DNA cloned in a prokaryotic vector was followed and correlated to the DNA synthesis inducing activity. Early region E1A proteins present in cells 24 h after injection of Ad2 DNA were detected with an antiserum prepared in rabbits against a fusion product of the N-terminal 131 amino acids of the Ad12 E1A proteins and the procaryotic β-galactosidase expressed in bacteria (Scott *et al.*, 1984). In contrast to SV40 T-antigen, expression of Ad2 proteins could not be directly correlated with cellular DNA synthesis (Figure 3). Most of the cells with strong nuclear radioactive label due to prolonged DNA synthesis did not show simultaneous expression of E1A proteins. In cells just having started to synthesize DNA and therefore showing weak nuclear label, expression of E1A proteins could be detected. A similar, but even more pronounced dissociation was repeatedly observed after microinjection of clones expressing either of the major E1A mRNAs, 12S or 13S mRNA (Figure 3). With an antiserum prepared against Ad12 E1A proteins, expression of E1A proteins from Ad2 constructs was dependent on the DNA concentration of the injected sample. A significant portion of the cells were stained by indirect immunofluorescence only at high DNA concentrations (1 mg/ml). Similar results were obtained with peptide antisera specifically reacting with the Ad2 E1A products (J.Lewis, in preparation). The efficiency of induction of DNA synthesis did not, however, vary with the DNA concentration (not shown).

Cells which at 24 h after injection contained high levels of E1A proteins usually had not entered the S-phase, whereas cells containing low or undetectable levels of protein had been induced to synthesize DNA (Figure 3). We failed to detect a larger number or a different proportion of brilliant and weakly stained cells at earlier or later times after DNA injections, suggesting that the E1A proteins may only be required in catalytic amounts for the induction of DNA synthesis. Thus, although E1A mRNA accumulation seems to be confined to the S-phase in transformed cells (Kao *et al.*, 1985), we did not find a strict correlation between antigen expression and DNA synthesis. Figure 3 also shows that no induction of cellular DNA synthesis was observed after

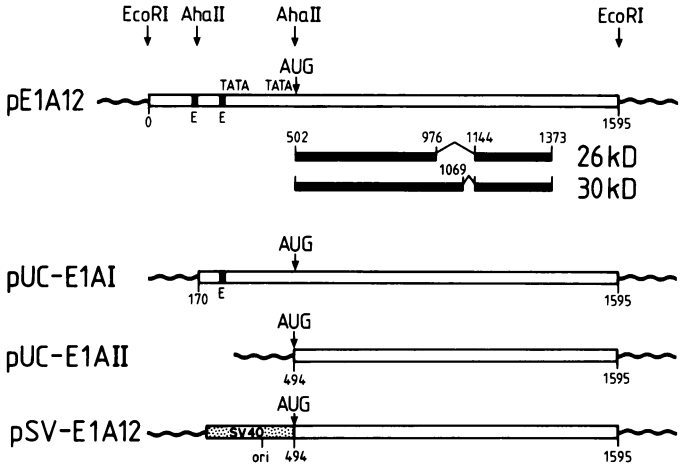

Fig. 4. Plasmids containing the early region E1A of Ad12. Construction of plasmid pE1A12 containing the left-most 1595 base pairs of the Ad12 genome was described by Bernards *et al.* (1982). Numbers indicate nucleotide positions from the left end of the Ad12 genome. The location of the TATA boxes, the enhancer elements (E) and the translational start signal (AUG) for the E1A proteins are indicated. Black bars represent the coding sequences for the two major E1A proteins. Plasmids pUC-E1AI and pUC-E1AII were constructed by excising Ad12 E1A sequences from pE1A12 at the *Eco*RI-linkers (Bernards *et al.*, 1982) and partially cleaving with *Aha*II. Fragments were inserted into the *Acc*I/*Eco*RI sites of plasmid pUC8 (Vieira and Messing, 1982) thereby establishing the two types of constructs. The plasmid pSV-E1A12 was constructed by exicising the E1A sequences from construct pUC-E1AII with *Hind*III and *Eco*RI and inserting them into the *Hind*III/*Eco*RI sites of plasmid pSV2-β-globin equivalent to pSV2-gpt (Mulligan and Berg, 1980). The polarity and size of the inserts were in each case confirmed by restriction mapping.

microinjection and expression of the mouse cellular *myc* gene. Like the cells expressing adenovirus E1A antigens only a fraction of the injected cells express the *myc* protein.

The Ad12 E1A region and induction of cellular DNA synthesis

Since all the Ad2 E1A constructs contained up to 8% of the left-most region of the Ad2 genome including part of the E1B region we also analysed induction of cellular DNA synthesis with an Ad12 E1A construct only containing the E1A transcription unit terminating at 4.5% of the viral genome (Bernards *et al.*, 1982). The constructs tested are shown in Figure 4 and include two truncated forms of the E1A transcription unit in plasmid pUC8 (Vieira and Messing, 1982). The construct pUC E1AI contains only one of the two enhancer elements identified in the Ad12 EA region (Hearing and Shenk, 1983); in pUC E1AII the E1A sequence starts with the translation initiation site of the 13S and 12S mRNA products (Sugisaki *et al.*, 1980).

Table II shows that the intact Ad12 E1A region induces DNA synthesis in ~22% of the cells injected comparable to the results obtained with Ad2 constructs (Table I). When both the truncated forms were analysed by microinjection, induction of DNA synthesis was observed as long as the E1A sequences were inserted in the plasmid DNA. To exclude that the latter results were due to promoter sequences in the bacterial plasmid, we also injected the purified DNA fragments containing the truncated E1A region excised from the plasmids. While the fragment containing one instead of two enhancer elements still induced DNA synthesis in 26% of the cells, the response with the DNA fragment truncated down to the E1A translation start was reduced to ~8%. These results suggest that the E1A protein(s) are responsible for the induction of cellular DNA synthesis in arrested cells.

In addition we substituted the Ad12 E1A promoter with the SV40 early promoter to study whether the lack of correlation between cellular DNA synthesis and accumulation of E1A products described previously was due to a promoter effect or a rapid turnover of the E1A proteins. The construct pSV-E1A12 shown in Figure 4 was tested in the microinjection assay. Though expression of E1A proteins from the SV40 promoter seemed to be more efficient, the number of cells which entered S-phase after microinjection of the plasmid did not increase significantly (Table II). The efficiency of induction of cellular DNA synthesis in quiescent cells by region E1A is therefore probably not simply a function of the expression level of E1A proteins. When expressed from the SV40 early promoter E1A proteins retain their low-level profile after cells started to synthesize DNA, suggesting that this effect is not due to transcriptional regulation of the adenovirus promoter, but might reflect a turnover of E1A proteins prior to induction of DNA synthesis.

Table II. Induction of DNA synthesis after microinjection of plasmids containing the E1A region of Ad12

DNA injected	Number of cells injected	Cells expressing E1A proteins (%)	Cells induced to synthesize DNA (%)
pE1A12	900	41	22
pSV-E1A12	400	67	28
pUC-E1AI	300	n.d.	28
Isolated fragment	400	n.d.	26
pUC-E1AII	300	n.d.	31
Isolated fragment	400	n.d.	8

n.d. = percentage not determined.
Constructs are described in Figure 4. Cells were injected with 100 μg/ml or 1 mg/ml of DNA. Cultures were labelled with 1 μCi/ml [^{3}H]thymidine for 24 h. Cells were fixed, stained for Ad2 E1A antigens by indirect immunofluorescence and exposed for autoradiography. The proportion of injected cells expressing E1A antigens and synthesizing DNA was determined.

Discussion

A simple assay system has been used for a functional analysis of genes involved in induction of DNA synthesis of growth-arrested cells. The present results establish that microinjection of plasmids containing the adenovirus E1A region can induce cellular DNA replication in growth-arrested cells; however, we have not established that the induced cells can undergo subsequent division. Instead of using serum starvation (possibly affecting steps other than growth control) cells were arrested in platelet-poor plasma to ascertain that only PDGF is required for the induction process. We have established in separate experiments (not shown) that >90% of the cells under these conditions respond with DNA synthesis after an exposure to 25–50 ng of PDGF per 10^6 cells for 4 h.

When several different plasmid DNA constructs of the E1A region of adenovirus type 2 (Table I) were microinjected into quiescent cells, plasmids expressing the 12S or 13S mRNAs induced cellular DNA synthesis, while those able to express 9S mRNA were inactive and so were repeated injections with the carrier vectors. These results are at variance with those reported by Spindler *et al.* (1985) who concluded that only expression of 12S mRNA from a virus background could induce cellular DNA synthesis in growth-arrested cells. A gene dose effect due to microinjection or different modes to bring about arrest, may explain the difference. We cannot, on the other hand, rule out that minute amounts of 12S mRNA were made from the 13S

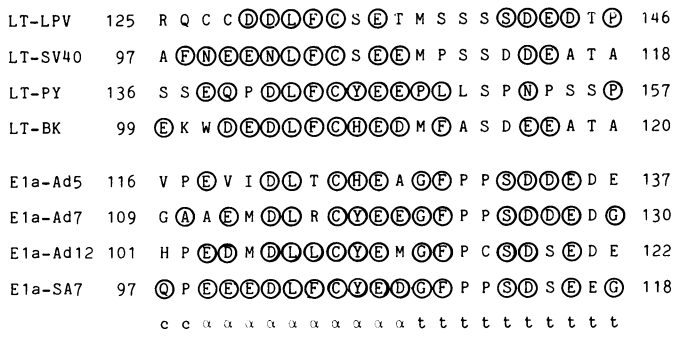

Fig. 5. Alignment of sequence segments from several adenovirus E1A proteins (adenovirus type 5, 7, 12 and SA7) and polyomavirus large T antigens (LT) including lymphotrophic papovavirus (LPV) SV40, BK, and mouse polyomavirus (PY). The complete sequences for the E1a proteins are listed by Kimelman *et al.* (1985) and for the polyomaviruses by Pawlita *et al.* (1985) excepting BK (Seil *et al.*, 1979). The mean secondary structure prediction over the aligned sequences, following the procedures and rules discussed by Zalkin *et al.* (1985), are given as (α) for helix, (t) for turn configuration, and (c) for coil. Conservative residue substitutions between sequence sets are circled. Since the secondary structures predicted are generally on the protein surface these regions may be available for DNA association. The first and last sequence positions of the respective segments are given. The entire National Biomedical Research Foundation sequence data bank (1984), consisting of 526 120 residues distributed over 2675 proteins, was searched for the following residue pattern: [N,D]L.C.E.....[D,N,E,] where (.) is any residue and brackets enclose amino acid alternatives at a single position. The pattern was selected such that all eight sequences contained conserved residues at a particular alignment position. The search yielded no other proteins displaying the pattern. A mean correlation coefficient over six residue physical characters thought to be important determinants of protein folding (helix, strand, and turn conformational preference parameters as well as hydrophobicity and polarity measures [see Argos *et al.* (1983) for a discussion] was determined for all cross-set alignments of which there are 16. The final mean correlation was 0.33 for the 16 comparisons with the maximum at 0.61 between LT-PY and E1A-SA7. These coefficients were tested for significance by comparing sequence alignments from 18 proteins whose three-dimensional structures are known to be unrelated. The 1−22 and 50−71 residues (22 positions corresponding to the alignment lengths in the E1A-LT comparisons) of the 18 proteins yielded 36 segments which were aligned as such and whose six-characteristic correlations were calculated over all 630 pair-wise comparisons. The mean was 0.00, the maximum 0.41, and the standard deviation (δ) 0.15. It is clear that the E1a-LT mean and maximum correlations are 2.2 δ and 4.1 δ above the mean coefficient of the control set.

mRNA specific DNA clones. However, since pm975 constructs with a mutated donor splice site for the 12S mRNA were used both by us and by Spindler *et al.* (1985), this explanation seems unlikely.

When we tried to correlate the expression of the microinjected genes with capacity to induce DNA synthesis, several cells showing brilliant immunofluorescence with an E1A-specific antibody failed to replicate cellular DNA although cells with less protein were triggered to synthesize DNA. In the positive controls with SV40 DNA there was as previously demonstrated (Mueller *et al.*, 1978) a good correlation between expression and induction of DNA synthesis. The lack of correlation between protein expression and induction of DNA synthesis may indicate that the DNA itself by titration of a cellular factor, may uncouple the G_0 block or that RNA or an unidentified protein product from this region could exert a controlling function. A more detailed study of the E1A region was therefore performed with the Ad12 E1A region, since the Ad2 DNA clones contained sequences beyond the polyA addition site of the E1A region and part of the E1B region. The Ad12 E1A clone (Bernards *et al.*, 1982) was truncated to eliminate one or both of the enhancer regions. The results (Table II) establish that induction of DNA synthesis is significantly reduced with fragments only containing the protein reading frame. We conclude from these results that the expression of the protein is probably of importance and that the E1A region is sufficient for induction of DNA synthesis.

We cannot rule out that region E1B present in clone pKGO170 can enhance the effect of region E1A since we observed more efficient induction with this clone than with the clones containing only 8% of the left-hand end of the Ad2 genome (Table I).

In contrast to SV40 virus DNA, where 60−80% of the arrested cells are induced, only 30−50% are induced after injection of adenovirus DNA. The difference may partially depend on the presence of foreign vector DNA, since the SV40 DNA is only effective in ~50% of the cells when integrated in a vector (Table I). Several attempts to enhance the induction of adenovirus DNA by introducing SV40 or other promoter elements in front of the gene showed no increase, suggesting that only a fraction of the cells are sensitive to the adenovirus signal. Under these conditions the *myc* gene whether under an SV40 (Table I) or an LTR promoter (not shown) failed to induce DNA synthesis in a significant portion of the cells suggesting that *myc* cannot replace the E1A region in this system and that *myc* expression by itself may not constitute an early triggering step in growth induction as recently suggested (Armelin *et al.*, 1984).

Baserga and his co-workers (Rossini *et al.*, 1981) have previously established that additional early regions of the adenovirus genome must be microinjected to induce DNA synthesis in temperature-sensitive cell mutants arrested at the permissive or non-permissive temperature. Both the E1A and the E2A regions seem to be required for induction of DNA synthesis at the permissive temperature in contrast to our results. Serum starvation and the use of a hamster cell line may account for a requirement of the E2A region coding for a DNA binding protein. At the non-permissive temperature the E1B and the E2B regions were required in addition to the E1A and the E2A regions. These latter findings raise the interesting possibility that the adenovirus gene products from the E1B and E2B regions can complement the proteins affected by the *ts* mutations in these cells, a hypothesis deserving further attention.

As both the 13S and 12S cDNA were effective in inducing DNA synthesis we searched for a common denominator between the coding sequence of the 12S mRNA of the E1A region of adenovirus and the papovavirus large T-antigens both of which can induce cellular DNA synthesis in arrested cells (Mueller *et al.*, 1978; Spindler *et al.*, 1985, and this paper). A computer search for sequence homology was performed according to a published comparison method (Argos, 1985) based on physical characteristics of the amino acids. Figure 5 identifies a homologous region in the large T-antigens of several papovaviruses and the 12S mRNA product from several adenoviruses which encompasses five strongly conserved residues including a cysteine, and spans a generally conserved length of 22 residues. This region lies between amino acids 97 and 118 in the SV40 large T-antigen and has not previously been identified as necessary for induction of DNA synthesis in arrested cells (Soprano, 1984). Detailed analyses of the capacity of SV40 deletion mutants to induce DNA synthesis in arrested cells (Soprano, 1984) demonstrated instead that a region between amino acid 228 and 274 is required to emit the induction signal, but no mutants upstream of amino acid 124 were analysed in this study. It is therefore possible that the identified region plays a role in the DNA synthesis response provided that the induction process requires two regions of the SV40 T-antigen, one around amino acid 100 and the other around amino acid 250. Site-directed mutagenesis in the homology area of the E1A 12S mRNA and the

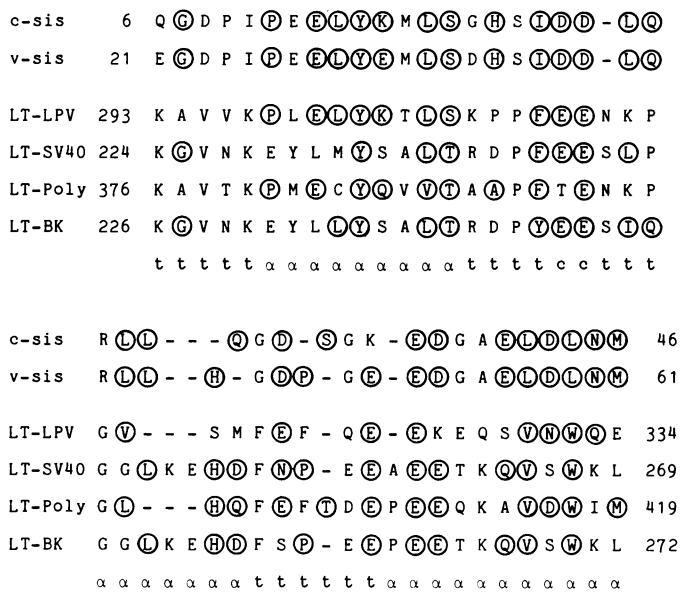

Fig. 6. Alignment of sequence segments from several adenovirus large T antigens as in Figure 5 and two PDGF-related transforming proteins [human c-*sis* (Josephs *et al.*, 1984) and simian sarcoma virus v-*sis* (Devare *et al.*, 1983)]. The annotations in the figure are as explained in the legend of Figure 5. The predicted structure indicates the possible exposure of these sequence regions on the protein surface. The sequence data bank was searched for the following pattern: [L,M,C]Y..[L,V][S,T]...[I,F,Y].[D,E] as explained in the legend of Figure 5. No other proteins were found displaying this pattern. Mean correlation coefficients over six residue characteristics between any one of the two *sis* proteins and any one of the LT proteins displayed an average of 0.35 and a maximum of 0.43 between v-*sis* and LT-SV40. The coefficients were tested for significance as explained in Figure 5 except that residue positions 1–45 and 50–95 were taken from each of the 18 control proteins, lengths which corresponded to that of the *sis*-LT alignments. The control protein mean, maximum and standard deviation (δ) were 0.00, 0.26 and 0.10, respectively for the 630 pair-wise comparisons. It is clear that the *sis*-LT mean and maximum correlations are 3.5 δ and 4.3 δ above the control mean coefficient.

large T-antigen (Figure 5) may provide a clue as to the involvement of these regions in induction of DNA synthesis in arrested cells.

It is interesting that the experimentally identified region (Soprano, 1984) in the large T-antigen, amino acids 228–274, shows conservation (Figure 6) with the sequence of the human and viral *sis* gene known to be homologous with the sequence of PDGF (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983), but not to any other known growth factor sequence. This finding may indicate that two domains in the large T-antigen may be required for induction of DNA synthesis; one structurally similar to the adeno E1A and the other similar to the *sis*. In addition to the two regions discussed above, a region between coordinate 0.39 to 0.33 map units corresponding to amino acids 332–432 is required for induction of ribosomal RNA synthesis in arrested cells as revealed by reactivation of mouse ribosomal RNA synthesis in a human-mouse hybrid cell lines (Soprano, 1984).

A fourth region encompassing the C-terminal 150 amino acids is obviously necessary for the adenovirus helper function in monkey cells which do not support adenovirus replication in the absence of a SV40 helper (Fey *et al.*, 1979). A fifth region encompassing amino acids 83–270 seems to be required for SV40 DNA binding (Rigby and Lane, 1983). The presence of multifunctional tumour antigens in the DNA tumour viruses compared with one or two cellular oncogenes in the RNA tumour viruses raises the possibility that the former have acquired some cellular genes advantageous for cellular replication through evolution due to the restriction imposed by their minimal genome size.

It is possible, however, that the DNA viruses may have kept only minimal functional domains capable of providing the necessary functions while the rest of the peptide backbone has been deleted to allow for rapid DNA replication. If so the DNA virus tumour antigens may be derived from several cellular genes, but only a fraction of each gene has been retained. In accordance small DNA fragments from SV40 DNA (Conrad and Botchan, 1982) and adenovirus DNA (U.Pettersson, personal communication) have been shown to hybridize to cellular DNA. This suggestion is supported by recent observations of sequence homology between exon segments of the LDL receptor, complement C9, EGF precursor, and blood coagulation factors (Südhof *et al.*, 1985), suggesting exon shuffling between the genes (Gilbert, 1985). The homologous epitopes observed here could be generated in a similar way. If functional epitopes really exist in the DNA virus tumour antigens, they may provide useful models for structural studies and for the design of artificial microgenes which may express a relevant function.

Materials and methods

Cell culture

NIH 3T3 mouse fibroblasts clone 7 (originally from Dr. D.Lowy, NIH, Bethesda, MD) were obtained from Dr. R.Müller, EMBL, Heidelberg. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum at 7% CO_2. The cells were never allowed to reach confluency and were kept in culture not longer than 2–3 months. After this period new stocks of cells were thawed.

To arrest cells in the G_0-phase for microinjection, 4×10^5 cells per 35 mm dish were seeded on 10 × 10 mm coverslips in DMEM containing 1% fetal calf serum. After 24 h the medium was changed to DMEM supplemented with 2.5% bovine platelet-poor plasma kindly provided by Dr. A.Habenicht, University of Heidelberg. After another 36–48 h, cells were used for microinjection. Purified platelet-derived growth factor (PDGF) was a gift from Dr. C.-H.Heldin, University of Uppsala, Sweden.

Adenovirus and plasmid DNA

Ad2 was grown in HeLa suspension cultures maintained with SMEM with 10% calf serum. Virus was isolated and purified by 2 cycles of CsCl density gradient centrifugation. Ad2 DNA was extracted as described (Doerfler *et al.*, 1972). Recombinant plasmids were propagated in HB101 or RRIΔM15 bacteria (Rüther, 1982). Plasmid DNA was prepared according to the method described by Birnboim (1983). A few plasmid preparations were purified by equilibrium centrifugation through CsCl gradients. Agarose gel electrophoresis, restriction enzyme digestion and transformation were performed according to standard methods (Maniatis *et al.*, 1982). Plasmid DNA was precipitated with ethanol and resuspended either in phosphate-buffered saline (PBS) or in 10 mM Tris-HCl pH 7.4, 10 mM EDTA (TE) at concentrations of 100 μg/ml or 1 mg/ml.

Microinjection

Cells were grown on 10 × 10 mm coverslips with marked areas. For injection the coverslips were transferred into a metal chamber with a glass bottom containing DMEM which could be kept at 37°C (developed by Dr. W.Ansorge, EMBL, Heidelberg) in order to exert as little stress as possible on the cells.

Injections were performed with the improved system for microinjection described by Ansorge (1982). In marked areas of the coverslip the nuclei of 50 cells were injected with ~10^{-11} ml of DNA solutions containing 100 μg or 1 mg DNA/ml of PBS or TE.

Injections of 100 cells took about 10–15 min. After injection, the coverslips were transferred back into the original dish and 1 μCi/ml [^{3}H]thymidine (Amersham 52 Ci/mmol) was added for different lengths of time, usually for 24 to 48 h.

Immunofluorescence

Detection of adenovirus E1A antigens. Cells were fixed with 3% paraformaldehyde in PBS at room temperature, permeabilized with 0.2% Triton X-100 in PBS at room temperature and incubated at 37°C with a 1:10 dilution of a rabbit antiserum raised against an Ad12 E1A-β-galactosidase fusion protein expressed in bacteria (Scott *et al.*, 1984) kindly provided by Dr. R.Ricciardi, The Wistar Institute, Philadelphia, PA.

Antipeptide antibodies against the E1A products of adenovirus type 2 were kindly provided by Dr. J.Lewis, Fred Hutchinson Center, Seattle, WA.

Reacted antibodies were visualized by incubation at 37°C with rhodamine-

labelled goat anti-rabbit immunoglobulins provided by Dr. T.Kreis, EMBL, Heidelberg.

Detection of murine myc protein. Cells were fixed as for detection of E1A antigens, incubated with 1:40 dilution of a rabbit polyclonal antiserum against human *myc* protein expressed in bacteria kindly provided by Dr. K.Moelling, University of Berlin. Rhodamine-labelled goat anit-rabbit immunoglobulins were used as second antibody.

Detection of SV40 T-antigen. Cells were fixed with acetone at −20°C, incubated first with a 1:50 dilution of a mouse monoclonal T-antigen antiserum provided by Dr. A.Poley, Centre for Molecular Biology, Heidelberg, and then with fluorescein-isothiocyanate-labelled rabbit anti-mouse immunoglobulins. Fluorescent cells were photographed under u.v.-light on a Zeiss inverted light microscope.

Autoradiography

Coverslips that had been processed for indirect immunofluorescence were mounted on slides and covered with autoradiographic stripping film Kodak AR10 according to the instructions of the manufacturer. Slides were exposed for 2 − 3 days, developed, stained with a 1:20 dilution of Giemsa's staining solution and the injected areas were photographed under a microscope.

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