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Genetics

Regulated expression of *ras* gene constructs in *Dictyostelium* transformants

(gene regulation)

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ABSTRACT **Constructs were made in which ≈500 base pairs of the 5′ flanking region of the *ras* gene of *Dictyostelium discoideum* and variable amounts of the coding region were linked to a ras cDNA in a transformation vector. These constructs then were used to transform *Dictyostelium* cells and their regulation was examined. In *Dictyostelium* transformants, transcripts from the *ras* gene constructs were found at high levels in cells in fast-shaking cultures containing cAMP, whereas transcripts were either not detectable or present at very low levels in cultures lacking exogenous cAMP. In slow-shaking culture, a significantly lower level of ras RNA was detected. When normal developing aggregates were dissociated, RNA from the *ras* constructs decreased rapidly but then reaccumulated in the presence of cAMP. These results show that the sequences necessary for the response to external cAMP are present within an ≈650-base-pair region upstream from the ATG start codon and/or within portions of the protein-coding region. Moreover, the proper regulation of *ras* gene expression in high-copy-number transformants suggests that *trans*-acting factors which may control transcription are not limiting. Vector constructs were also examined in which the cDNA was present in the opposite orientation compared to the gene fragment (antisense orientation). When these were transfected into cells, no transformants were obtained, suggesting that expression of the *ras* gene is essential for vegetative growth.**

The transforming potential of the *ras* oncogenes was initially recognized in retroviruses (1–4), and since then, their cellular counterparts have been found in species ranging from mammals (5–7) and *Drosophila* (8) to yeast (9, 10) and *Dictyostelium* (11). The ras protein has been shown to bind guanine nucleotides (12, 13) and to carry an intrinsic GTPase activity (14). Mutant *ras* genes capable of transforming mammalian cells code for proteins with decreased GTPase activity (15). In yeast, ras protein is essential for growth and sporulation (16, 17) and has been implicated as a regulatory protein of adenylate cyclase (18).

The *ras* gene in *Dictyostelium discoideum (Dd-ras)* was originally identified as a cell-type-specific gene that was expressed in migrating pseudoplasmodia in prestalk cells but not in prespore cells (11). It is also expressed in vegetative cells, but the ras mRNA disappears rapidly upon initiation of development. The gene encodes two different mRNAs that are differentially regulated (11). ras protein is present in vegetative cells and during early development. The ras protein level in whole aggregates then decreases during later development and is found in prestalk but not prespore cells (refs. 11 and 19 and unpublished observations). Previous studies also showed that the endogenous *ras* gene, like other genes expressed in prestalk cells, can be induced by cAMP in shaking culture in the absence of sustained cell–cell contact (20). Moreover, when developing aggregates are dissociated, the levels of the two ras mRNAs rapidly decrease and can be reinduced by the addition of cAMP (11).

In eukaryotes, *cis*-acting regulatory regions have been shown to lie within the coding region as well as in the 5′ flanking region of genes and, in one case, 3′ of the gene itself (21–23). Previously, we have shown that ≈600 base pairs (bp) of 5′ flanking sequence are sufficient for proper transcriptional regulation of the *actin-6* gene in *Dictyostelium* transformants (24). To determine whether the *Dd-ras* gene is properly regulated in transformants and to locate the *cis*-acting sequences necessary for its proper regulation, we constructed a set of vectors carrying ≈550 bp of 5′ flanking region and various portions of the coding region. A ras cDNA was fused to these gene fragments out of frame to eliminate the overproduction of a functional ras protein, which might be deleterious to the cells. These constructs were used to transform *Dictyostelium* cells, and the regulation of expression of the *ras* gene constructs was studied. We show that these gene constructs are regulated by cAMP in a manner similar to the endogenous *ras* gene and, thus, that the sequences necessary for proper regulation in a relatively short region 5′ of the ATG translation initiation codon and/or within portions of the coding region. We also attempted to transform *Dictyostelium* with constructs carrying the cDNA linked to the *ras* promoter in an antisense polarity. Transformation with vector constructs that encode antisense mRNA has been found to substantially reduce the expression of the corresponding endogenous or transfected genes (refs. 25–27 and unpublished data). The failure to obtain transformants with an antisense construct of *Dd-ras* strongly suggests that, as in yeast (16), *ras* gene expression is essential for vegetative growth in *Dictyostelium*.

MATERIALS AND METHODS

Vector Constructions and Sequencing. The polylinker from pUC7 (28) was inserted into the *Eco*RI site of B10 (24). The *actin-6–Neo*^{R} fusion gene was then excised at the *Bam*HI sites flanking the gene fusion and ligated into the *Bam*HI site of pBR322. The resulting plasmids with the two possible orientations were named B10A (with the 5′ end of *actin-6* proximal to the pBR322 *Eco*RI site) and B10B (with the opposite orientation).

Construct A_1 in B10A was made as follows: the *Cla* I–*Rsa* I fragment, containing the prospective promoter from the *Dd-ras* gene (see Fig. 1 and ref. 11) was isolated. The cDNA clone *Dd-ras* c1 (11) was inserted into the *Pst* I site of M13

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Abbreviations: *Dd-ras*, *ras* gene of *Dictyostelium discoideum*; bp, base pair(s); kb, kilobase(s).

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mp9 and then excised at the *Hin*cII and *Hin*dIII sites of the M13 mp9 polylinker (which are located 5′ and 3′ of the cDNA insert, respectively) and purified. The cDNA and *Cla* I–*Rsa* I fragments then were ligated into B10A digested with *Hin*dIII/*Cla* I. This construct then was used to transform *Escherichia coli.*

For construct A_2, *Dd-ras* was digested with *Hin*fI, the ends of the fragments were filled in with DNA polymerase I (Klenow fragment), and the resulting blunt-ended molecules were digested with *Cla* I. The *Cla* I-blunt-ended *Hin*fI fragment was purified and ligated into B10A together with the *Hin*dIII–*Hin*dII fragment containing the cDNA, yielding construct A_2. For A_3, *Dd-ras* was digested with *Pst* I; the DNA was blunt-ended with T4 DNA polymerase and then digested with *Cla* I. The *Cla* I-blunt-ended *Pst* I fragment was purified and ligated into B10A along with the cDNA fragment to yield construct A_3

Constructs of the B series (B_1, B_2, and B_3) were obtained by similar ligations using the M13 mp9 clone containing *Dd-ras* cDNA inserted in the opposite orientation. The *Dd-ras* c1 orientation and sequence at the junction with the 5′ gene fragments were determined by DNA sequencing.

Dictyostelium Transformation. The B10A constructs were reintroduced into *Dictyostelium* cells by the calcium phosphate coprecipitation method as described (24). The selection of transformed cell populations and single colonies capable of growing in the presence of antibiotic G418 at 20 μg/ml is described in the text.

DNA and RNA Preparation. DNA and RNA were extracted from transformed cells, electrophoresed in agarose gels, transferred to nitrocellulose, and probed with the purified cDNA insert from *Dd-ras* c1 as described (11, 20, 24). $Poly(A)^+$ RNA was purified by poly(U)-Sepharose chromatography.

Culture Conditions. Shaking culture conditions are essentially as described (11, 20). Vegetative cells were harvested, washed, and placed in fast (230 rpm) or slow (70 rpm) shaking culture in phosphate-deficient Mes-buffered medium containing 1 mM EDTA. After 6 hr, cAMP was added (final concentration, 300 μM) to a fast-shaking culture. Cells were harvested at specific times (see Fig. 4), and RNA was isolated. Examination of *ras* gene regulation in developing cells after disaggregation is described in the legend to Fig. 4.

RESULTS

***ras* Gene Constructs.** We wanted to determine whether *ras* gene constructs transferred into *Dictyostelium* cells would be regulated in a similar manner as the endogenous gene under several physiological conditions. Constructs were made that contained potential *cis*-acting regulatory regions, including the 5′ flanking region and sequences situated within introns and protein-coding regions. We fused fragments of varying length from the 5′ region of the genomic clone to a *Dictyostelium ras* cDNA clone (11) so that the reading frame was lost and the constructs would encode either no functional protein or only a small part of the most NH_2-terminal region of the ras protein (Fig. 1).

All constructs start at the *Cla* I site 642 bp upstream from the ATG initiation codon (Fig. 1). The *Cla* I–*Rsa* I fragment (545 bp) in the construct A_1 contains part of the 5′ noncoding region but not the initiation codon. The *Cla* I–*Hin*fI fragment in the construct A_2 contains the first exon, the first intron, and 4 bp of the second exon of the genomic DNA fused to the cDNA. The presence of 4 bp from M13 mp9 at the junction, however, introduces a frame shift, thus resulting in a fusion gene that contains the first 26 codons of the *ras* gene, followed by 10 out-of-frame codons and a stop codon (data not shown). The *Cla* I–*Pst* I fragment in the construct A_3 extends over the first three exons and ends in the fourth exon of the *Dd-ras* gene. Sequencing showed that this fusion gene encodes the first 58 amino acids of the ras protein and 7 additional amino acids in a different reading frame (data not shown). A second series of constructs (B_1, B_2, and B_3) were made in which the cDNA was inserted downstream from the 5′ regions in the opposite or antimessage polarity (Fig. 1, cDNA insert in opposite orientation of that shown).

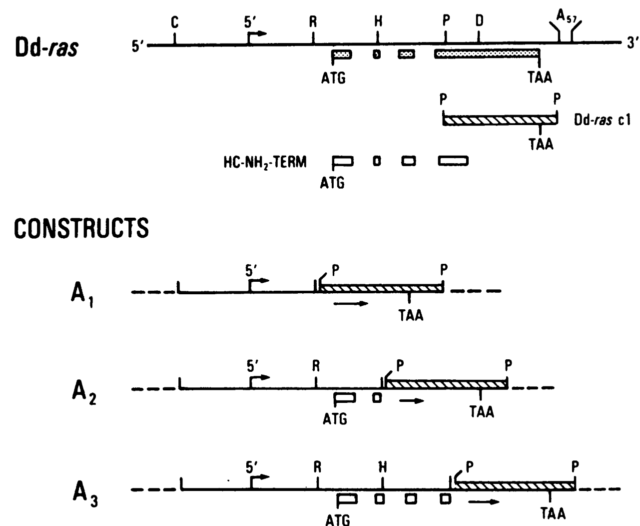

FIG. 1. Map of *ras* gene A-series constructs. Construction of the vectors is described in *Materials and Methods*. A map of the cloned endogenous *Dd-ras* gene (11) is shown, with the regions coding for the ras protein indicated by stippled bars. Regions coding for the highly conserved (HC) NH_2-terminal portion of the protein are represented by open bars. The map of the *Dd-ras* c1 cDNA (hatched bars) is shown in relationship to the genomic sequence. The putative 5′ start of the mRNA (bent arrow) and the ATG (initiation) and TAA (termination) codons of the protein are indicated. A_{57}, adenylate homopolymer. Restriction sites: C, *Cla* I; R, *Rsa* I; H, *Hin*fI; P, *Pst* I; D, *Dde* I.

The fusion genes were cloned in the B10A transformation vector resulting in a tandem arrangement of the *ras* gene fusion (5′) and the *actin-6–Neo^R* fusion (3′) (see *Materials and Methods*). Independent experiments (data not shown) showed that the *actin-6* 5′ upstream region contains a strong transcription termination signal. We therefore expected a *ras* RNA of defined size terminating in the *actin-6* 5′ sequence.

The A-series constructs were introduced into *Dictyostelium* and transformants were selected for resistance to antibiotic G418 (24). Transformed populations were first grown in the absence of G418 and DNA was isolated. Growth of cells transformed with B10 derivatives in the presence of G418 results in the selection of stable transformants having a high copy number (50–200) of vector sequences per cell (unpublished data). The populations grown in the presence of G418 (20 μg/ml) were plated at low density on *Klebsiella aerogenes* to isolate single colonies. DNA isolated from transformed populations or from single colonies was digested with *Cla* I plus *Hin*dIII, which excises the *ras* gene constructs. The DNA was then size-fractionated by electrophoresis in agarose gels, blotted onto nitrocellulose, and probed either with labeled pBR322 DNA, which identified the B10A vector (data not shown), or with a *Dd-ras* c1 cDNA probe (Fig. 2). DNA from populations (lanes Pop) or single colonies (lanes Single) derived from cells transformed with A_1, A_2, or A_3 showed fragments of 1.0, 1.3, and 1.6 kb, respectively, each corresponding to the size of the *ras* fusion construct (compare with plasmid DNA). From comparison of the relative hybridization intensity of the *ras* cDNA to the endogenous gene, we estimate that the copy number of the *ras* fusions in

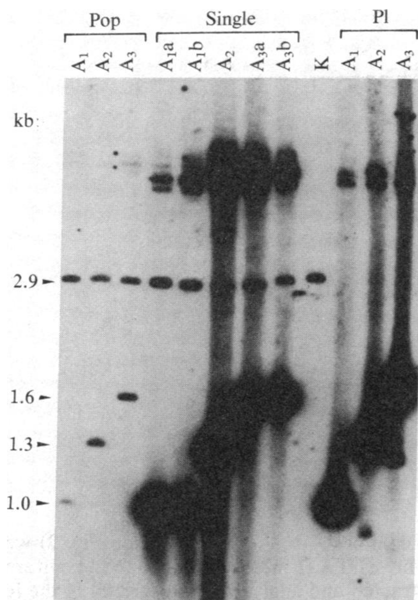

FIG. 2. Analysis of A-series vector DNA in *Dictyostelium* transformants. DNA was isolated from cell populations shortly after transformation (lanes Pop) or from single colonies (lanes Single; A_1a, A_1b, A_2, A_3a, and A_3b each represent individual colonies) selected for growth in the presence of antibiotic G418. Lane K: DNA from nontransformed cells. Lanes Pl: A_1, A_2, or A_3 plasmid DNA (samples equivalent to 200 copies per cell). DNA samples were digested with *Cla* I plus *Hin*dIII (which excises the *ras* gene construct), electrophoresed in a 1% agarose gel, blotted to nitrocellulose, and probed with *Dd-ras* c1 cDNA insert. The 2.9-kb band corresponds to the endogenous *ras* gene. Bands in the upper portion of the autoradiograph are due to partial digestion of DNAs. Overexposure of A-construct bands was necessary to see endogenous gene copy.

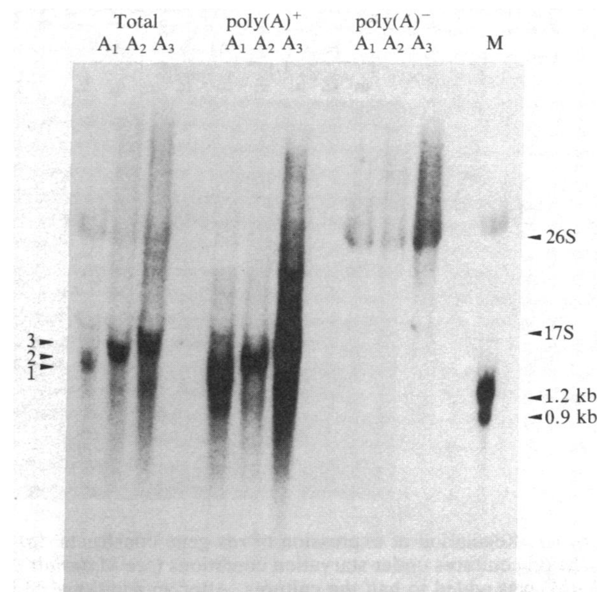

FIG. 3. RNA from vegetative transformed cells. RNA was extracted from cell populations grown in the presence of G418 to a density of $\approx 10^6$ cells per ml (lanes A_1, A_2, and A_3). Total (7.5 μg per lane), poly(A)$^+$ ($\approx$1 μg), or poly(A)$^-$ (10 μg) RNA was electrophoresed in a 1.2% agarose gel, blotted, and probed with *Dd-ras* c1 fragment. Since the poly(A)$^+$ was isolated by passing total cell RNA once over a poly(U)-Sepharose column, the efficiency of removal of rRNA was variable and thus it is not possible to directly compare the level of hybridization. Discrete bands ($\approx$1250, 1450, and 1550 bases, arrowheads 1, 2, and 3) can be seen in RNA samples from transformants A_1, A_2, and A_3, respectively. Total RNA extracted from Ax-3 cells from a slow-shaking culture was run as a marker and shows the size of the two *ras* gene transcripts [0.9 and 1.2 kilobases (kb)] found during development of nontransformed *Dictyostelium* (lane M). Only the 1.2-kb RNA is expressed in nontransformed vegetative cells. This RNA would run within the smear present in lanes carrying RNA from transformed cells and is not visible. Reprobing the same filter with different mRNA probes indicated that this smear was not due to RNA degradation (data not shown, see text).

populations of transformed cells varied, on the average, between one and four copies per cell. In contrast, colonies from cultures grown in G418 contained $\approx$180 copies per cell (Fig. 2, lanes Single and Pl). Because of the relatively low level of expression of the *ras* promoter, cell populations and single colonies carrying a high copy number of vector DNA were used for subsequent analysis of expression.

Expression of the *ras* Gene Constructs During Vegetative Growth. RNA was isolated from vegetative cultures derived from clones carrying the A_1, A_2, or A_3 constructs, electrophoresed in agarose gels under denaturing conditions, transferred to nitrocellulose, and probed with the ras cDNA (Fig. 3). RNA of discrete sizes was observed with each of the three types of transformants. These RNAs differ in size and are about 50 times more abundant than the endogenous ras mRNA, as might be expected from the high copy number of the vector DNA. When the blot was probed with labeled pBR322 DNA, no hybridization to the A-series-specific RNAs was observed (data not shown), suggesting that the 5′ end of these RNAs lies within the 5′ flanking region present in each *ras* construct. The sizes of the three RNAs are consistent with termination occurring within the *actin-6* promoter region located 3′ of the *ras* gene fusion, as observed with other B10 constructs (unpublished data). As seen in Fig. 3, the transcripts are polyadenylylated as defined by binding to poly(U)-Sepharose. Two bands can be seen in several lanes (see also Fig. 4*b*), suggesting that either two different initiation or polyadenylylation sites are used. The results also show that the level of RNA from the A_2 and A_3 constructs is higher than that from the A_1 construct (Fig. 3).

Comparison of the size difference between the DNA fragments used in the A_1 and A_2 constructs (277 bp) and the resultant transcripts ($\approx$200 nucleotides) suggests that the first intron (102 nucleotides) in the A_2 transcripts is removed. Further indication that splicing occurs is obtained by comparing transcripts from A_1 and A_3 constructs, which show a size difference of 300 nucleotides, in comparison to the DNA fragments, which differ in size by 568 bp. This is in agreement with the removal of the two introns, which have a combined size of 295 nucleotides.

Regulation of the *ras* Gene Constructs by cAMP. We wanted to determine whether the *ras* gene constructs are regulated by cAMP in a manner similar to the endogenous *ras* gene and other prestalk-specific genes (11, 20). *ras* and other prestalk specific-genes but neither prespore-specific nor cell-type-nonspecific genes are induced when micromolar levels of cAMP are added to cells in a fast-shaking culture (20), conditions that eliminate sustained cell–cell contact. We examined the expression of the A-series constructs in transformed cells under these culture conditions. As shown in Fig. 4*a*, mRNA of the appropriate sizes is expressed in transformants carrying many copies of each vector in the presence but not in the absence of added cAMP. Cells in slow-shaking cultures accumulate much lower levels of the transcripts, as observed with the endogenous *ras* gene (ref. 11 and data not shown).

When normally developing multicellular aggregates are disaggregated, the levels of prestalk- and prespore-specific mRNAs rapidly decrease (20). When endogenous cAMP is added to these cells, the cell-type-specific mRNAs rapidly reaccumulate to levels between 50 and 100% of those found in normal developing aggregates (11, 20, 29). In the case of the

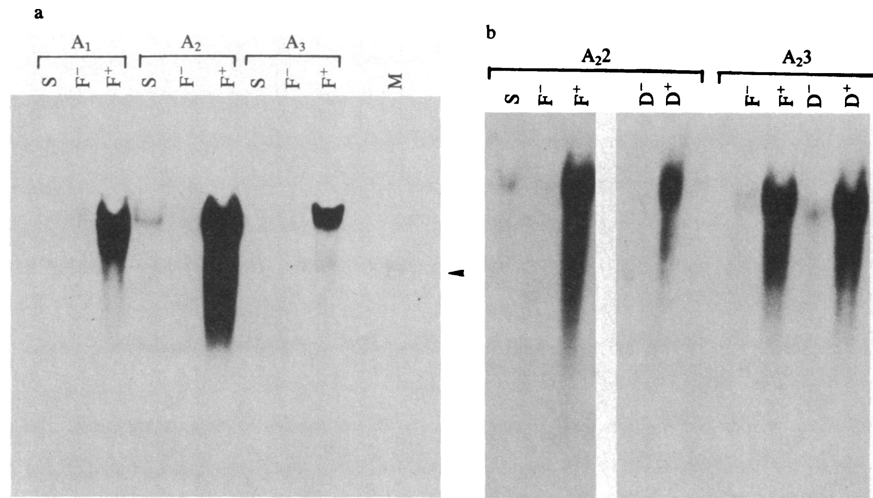

FIG. 4. Regulation of expression of *ras* gene constructs. (*a*) Cells from the single colonies A_1b, A_2, and A_3a (see Fig. 2) were placed in fast-shaking cultures under starvation conditions (see *Materials and Methods*). After 6 hr, EDTA (1 mM) was added to the cultures and cAMP (300 μM) was added to half the cultures. After an additional 13 hr, total RNA was extracted and analyzed as described in the legend to Fig. 3. A large increase in the ras RNA level can be seen in samples from the cultures to which cAMP was added (F^+) as compared to levels found in cultures without added cAMP (F^-) or in slowly shaken cultures (S). In lanes A_1, 10 μg of RNA was loaded, whereas 3 μg was used in lanes A_2 and A_3. Lane M: 10 μg of RNA from Ax-3 cells starved under fast-shaking conditions, with the addition of cAMP (300 μM). Arrowhead, migration of the endogenous ras transcript from fast-shaking cultures. (*b*) Vegetative cells from two single-colony isolates ($A_2$2 and $A_2$3) from a population transformed with construct A_2 were plated for development (20). At 18 hr (between the tight aggregate and early culmination stage), the cells were disaggregated into single cells and placed in fast-shaking culture for 3 hr. cAMP (300 μM) was given to half the cultures and total RNA was isolated from cells 2 hr later (see refs. 11 and 19 for details). RNA samples (7.5 μg) were analyzed as described in *a*. Lanes: S, slow-shaking culture; lanes F^+ and F^-, fast-shaking culture with and without added cAMP, respectively; D^+ and D^-, disaggregated cells with and without cAMP, respectively.

ras gene, one of the two mRNAs (0.9 kb) accumulates to normal levels while the other (1.2 kb) is induced to levels 10- to 20-fold above that found in normal aggregates (11). Similar experiments were performed on transformed cells carrying the A_1, A_2, and A_3 constructs (Fig. 4*b* and data not shown). Cells were allowed to develop until ≈18 hr (early culmination state) and then disaggregated. The dissociated single cells were placed in fast-shaking culture to eliminate sustained cell–cell interactions and after 3 hr, cAMP was added to half the cultures while the others were maintained in the absence of cAMP. After an additional 2 hr, the cells were harvested and the level of ras RNA was assayed. Fig. 4*b* shows the analysis of two single-colony transformants carrying the A_2 construct. Substantial amounts of A_2 transcripts were observed in cells from the culture receiving cAMP but not from control cultures. Similar results were obtained for cells carrying A_1 and A_3 constructs (data not shown). These results indicate that the transformed *ras* gene is properly regulated and that the sequences necessary for this regulation are present in the gene fusions.

Transformation with Antisense Constructs. Constructs B_1, B_2, and B_3 contain the *Dd-ras* c1 inserted so that an antimessage transcript is produced from the same promoter used in constructs A_1, A_2, and A_3, respectively. The B-series vectors were introduced into *Dictyostelium* cells and G418-resistant cells were selected by use of procedures identical to those used for the A-series transformants. In each case, the number of colonies surviving selection was <5, approximately the same as with the pBR322 control, in contrast to the 200–1000 colonies obtained with each A-series construct or with B10A alone in parallel experiments. Colonies that did come through the selection did not contain vector DNA and were unable to grow in the presence of G418 (data not shown). Even though these experiments yielded negative results (no transformants), they suggest that expression of the B-series constructs may be lethal, possibly due to the formation of double-stranded hybrids *in vivo* between the B-series transcripts and the ras mRNA and the subsequent inhibition of ras protein synthesis (25, 27).

DISCUSSION

We wanted to determine whether *ras* gene constructs inserted into *Dictyostelium* cells by DNA-mediated transformation are properly regulated when cells are given exogenous cAMP. Constructs were made in which ≈550 bp of the 5′ flanking region and portions of the 5′ untranslated and protein-coding region of the *Dictyostelium ras* gene were fused to a *ras* cDNA clone containing approximately two-thirds of the protein-coding region and the 3′ untranslated region. The A-series constructs transformed *Dictyostelium* cells with approximately the same frequency as control vector B10A (ref. 24 and unpublished data). When transformed populations were grown in the absence of G418, the cells contained, on the average, 1–4 copies of the vector DNA, as previously reported for the B10 vector (24). When single colonies were selected after growth in G418, the copy number was ≈180 and was similar for all the A-series vectors.

Analysis of the transcripts from the *ras* gene constructs in vegetative cells indicates that the transcripts probably initiate at the same promoter(s) in all the constructs and terminate preferentially in the 5′ flanking region of the adjacent *actin-6–Neo^R* fusion gene, which contains a putative termination signal (unpublished data). The presence of a smear extending from the RNA bands is not due to overall RNA degradation, as indicated from hybridization with an actin-specific probe (data not shown), but may be the result of a decreased stability of the transcripts and/or to the presence of minor termination sites at other positions. Transcripts from all three constructs were preferentially retained on poly(U)-Sepharose columns (Fig. 3). This could be due to correct posttranscriptional polyadenylylation or to the high adenylate content of parts of the noncoding regions.

We have also investigated whether the expression of the gene constructs is regulated by cAMP. As with the endoge-

nous *ras* gene (11), all three constructs are expressed at a very high level in fast-shaking cultures of starved cells and cultures of disaggregated cells that have received cAMP. A very low level of expression was observed in cultures lacking exogenous cAMP. The gene was expressed in aggregates produced in slow-shaking culture but at substantially lower levels, as is the case for the endogenous gene (11). Thus, the *cis*-acting regions essential for regulation by cAMP are present within the portions of the *ras* gene included in all three constructs. The regulatory regions are probably confined to the 5′ flanking region, unless *cis*-acting elements are present in the cDNA fragment. The relative levels of the RNAs accumulated in transformed vegetative cells varied with the different constructs (Fig. 2, total cell RNA) even though the copy number of vector DNA in all three constructs seemed similar in the single colonies. The A_1 construct, which yielded a lower level of RNA, lacks the normal ATG translation initiation codon of the *ras* gene and contains only a portion of the 5′ untranslated region. It is unclear whether this variation in mRNA levels may result from a higher level of transcription of the A_2 or A_3 constructs or to a lower level of stability of the A_1 transcripts.

Transcripts of the A-series constructs are present at an elevated level in vegetative and developing cells relative to the endogenous *ras* gene, as might be expected from the copy number of the vector DNA in these cells (unpublished data). Because the constructs are regulated in a manner similar to the endogenous gene under the experimental conditions examined, we assume that regulatory factors are not limiting. It should be noted that the *actin-6* promoter, which is driving transcription of the neomycin-resistance gene, shows a reduced level of expression in fast-shaking cultures or in disaggregated cells with added cAMP relative to the level in vegetative cells (data not shown). Thus the expression of the *ras* gene constructs appears to be independent of the linked *actin-6* promoter in these plasmids.

Two transcripts are produced from each *ras* gene construct. Introns appear to be removed correctly, as determined from the sizes of the A_2 and A_3 transcripts. Since only a single major transcript is detected from other gene constructs that terminate within the *actin-6* 5′ end (unpublished data), it is possible that the two mRNAs result from transcriptional initiation at two different sites. Two transcripts are produced from the endogenous *ras* gene during development, but the size difference between these (about 0.3 kb) (11) is larger than that observed for the two A_1, A_2, or A_3 transcripts.

In the B-series plasmids, the cDNA was inserted in the antimessage polarity. *Dictyostelium* transformations with B constructs resulted in no greater number of cells surviving G418 selection than control transformations with pBR322, and none of the survivors contained vector DNA on subsequent analysis. Since these constructs carry the same promoter, we believe that the DNA was transcribed in the cells to give RNA complementary to ras mRNA, thus inhibiting translation of the normal ras mRNA. This interpretation is consistent with recent observations that when a *discoidin-I* gene construct in which a portion of the protein-coding region is present in the antisense direction is transferred into *Dictyostelium* cells by transformation, the steady state level of discoidin I mRNA decreases from 0.5–1% of the total mRNA to barely detectable levels. These results suggest that the expression of the *ras* gene is necessary for vegetative growth in *Dictyostelium*, as has been shown to be the case for yeast (16, 17).

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